Crosstalk between Androgen and Epidermal Growth Factor Signalling in the Ovary

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Statement of Originality

All experiments included in this thesis were performed by the author unless otherwise stated and acknowledged in the text and figure legends. Where the work of others is quoted the source is always given.
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Abstract

The development and survival of preantral follicles within the mammalian ovary must be tightly regulated in order to ensure a healthy supply of oocytes for the entirety of a woman’s reproductive lifespan. A complex regulatory network of both stimulatory and inhibitory factors exists to control activation of primordial follicles and preantral follicle growth. This thesis aimed to investigate interactions between local intraovarian growth factor and steroid signalling pathways. The epidermal growth factor (EGF) family, previously known to be essential during ovulation, was shown to play a role earlier during preantral follicle development. ErbB receptor subtypes EGFR and ErbB2 as well as EGF-like ligands were expressed in isolated mouse preantral follicles. Cultured preantral follicles exposed to exogenous EGF ligand grew significantly larger than controls and displayed widespread alterations in the expression of genes and proteins essential to follicular development. Receptor inhibitor studies showed that EGF signalling between and within preantral follicles likely activates both EGFR homodimers and EGFR-ErbB2 heterodimers that converge on the MAPK cascade. Androgens were also shown to stimulate preantral follicle growth and alter expression of proteins key to follicular development in culture. Androgens signal primarily through the androgen receptor (AR) that acts classically as a ligand activated transcription factor, however it was shown that AR can also induce rapid non-genomic phosphorylation events in the MAPK cascade in mouse granulosa cells. Interactions between androgen and EGF signalling were uncovered in preantral follicles. Androgens regulate expression of ErbB receptors and EGF-like ligands in cultured preantral follicles. Interestingly, selective inhibition of either EGFR or ErbB2 attenuated the effect of androgens on follicle growth, indicating that ErbB activity is required for maximal androgenic stimulation. The mechanisms of androgen-EGF crosstalk remain unclear, however this novel interaction provides insights into the complex and interconnected regulatory networks within the ovary that control preantral follicle development.
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<td>17βHSD</td>
<td>17β-hydroxy steroid dehydrogenase</td>
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<tr>
<td>3βHSD</td>
<td>3β-hydroxy steroid dehydrogenase</td>
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<td>Anti-Müllerian hormone</td>
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<td>AMHR2</td>
<td>AMH type 2 receptor</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>Androgen receptor</td>
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<td>ARE</td>
<td>Androgen response element</td>
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<td>Areg</td>
<td>Amphiregulin</td>
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<tr>
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<td>Global androgen receptor knock out</td>
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<tr>
<td>bFGF</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
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<td>Bovine serum albumin</td>
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<td>Kit tyrosine kinase receptor</td>
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<td>Carboxymethyl-oxime</td>
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<td>cAMP response element binding</td>
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<td>Cytochrome P450 17</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>Abbreviation</td>
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<tr>
<td>DBD</td>
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<td>DHT</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
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<td>Early growth response 1</td>
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<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
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<td>Epigen</td>
</tr>
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<td>ErbB</td>
<td>Epidermal growth factor receptor family</td>
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<td>Ereg</td>
<td>Epiregulin</td>
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<td>Extracellular signal-regulated protein kinase</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
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<td>FBS</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>GA-binding protein</td>
</tr>
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</tr>
<tr>
<td>GCARKO</td>
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</tr>
<tr>
<td>GDF9</td>
<td>Growth differentiation factor 9</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>Abbreviation</td>
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<tr>
<td>GL</td>
<td>Granulosa lutein</td>
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<tr>
<td>GRB2</td>
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<td>HB-EGF</td>
<td>Heparin-binding EGF</td>
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<td>hCG</td>
<td>Human chorionic gonadotropin</td>
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<tr>
<td>HPG</td>
<td>Hypothalamus-pituitary-gonadal</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>Heat shock protein</td>
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<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
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<tr>
<td>IGF1R</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>ITS</td>
<td>Insulin transferrin sodium selenite</td>
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<tr>
<td>IVF</td>
<td>In vitro fertilisation</td>
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<tr>
<td>JAK-STAT</td>
<td>Janus kinase/signal transducers and activators of transcription</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
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<td>KGF</td>
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<td>KL</td>
<td>Kit ligand</td>
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<td>Ligand-binding domain</td>
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<td>LH</td>
<td>Luteinizing hormone</td>
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<td>LHCGR</td>
<td>LH/human chorionic gonadotropin receptor</td>
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<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
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<td>MEM-α</td>
<td>minimum essential medium alpha</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>NDS</td>
<td>normal donkey serum</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
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<td>Neuregulin</td>
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<td>NTD</td>
<td>N-terminal transactivation domain</td>
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<td>Oo</td>
<td>Oocyte</td>
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<td>OoARKO</td>
<td>Oocyte specific-androgen receptor knockout</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PCNA</td>
<td>Proliferation cell nuclear antigen</td>
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<td>PCOS</td>
<td>Polycystic ovarian syndrome</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>PGC</td>
<td>Primordial germ cell</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
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<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
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<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>POI</td>
<td>Premature ovarian insufficiency</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RALT</td>
<td>Receptor-associated late transducer</td>
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<td>RBD</td>
<td>Ras-GTP-binding domain</td>
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<tr>
<td>RGB</td>
<td>Red-Green-Blue</td>
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<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SH2</td>
<td>Src homology 2</td>
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<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex hormone-binding globulin</td>
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<tr>
<td>SHC</td>
<td>Src homology and collagen</td>
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<tr>
<td>SNAI</td>
<td>Snail family zinc finger</td>
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<tr>
<td>SOS1</td>
<td>Son of sevenless homolog 1</td>
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<td>SRD5A</td>
<td>Steroid 5α-Reductase</td>
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<td>Star</td>
<td>Steroidogenic acute regulatory protein</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>TBS-T</td>
<td>Tris-buffered saline-Tween</td>
</tr>
<tr>
<td>TCARKO</td>
<td>Theca cell-specific androgen receptor knockout</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Tfm</td>
<td>Testicular feminization mutation</td>
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<tr>
<td>TGF-α</td>
<td>Transforming growth factor-alpha</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Tissue inhibitor of metalloproteinase 1</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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</table>
Chapter 1: Introduction

1.1 The ovary

The ovary is a principal organ in the female reproductive system. The mammalian ovary serves two main physiological roles; the production of mature oocytes capable of fertilisation and the secretion of steroid hormones essential for female reproduction. The highly organised structure of the female gonad consists of follicles, which are the basic functional units, set in stroma and enclosed in the ovarian surface epithelium. Ovarian follicles are comprised of a single germ cell (oocyte), surrounded by supporting somatic granulosa cells (GCs), enveloped within a basal lamina. During the later stages of development a layer of theca interstitial cells also surrounds the follicle.

The ovary consists of a finite pool of follicles that serve the entirety of the female’s reproductive lifespan. At sexual maturation, the ovary commences the cyclical release of haploid oocytes for fertilisation (McGee and Hsueh, 2000). During ovulation, a surge of gonadotropin signalling stimulates the release of a single oocyte in mono-ovulatory species (e.g. human) or several oocytes in poly-ovulatory species (e.g. mouse). The finite number of oocytes in the ovary depletes with age and in a few species, including humans, the lack of functional oocytes is marked by entrance into the menopause, defined as the permanent cessation of ovulation.

The synthesis and secretion of steroids by the ovary is essential throughout reproductive life. Ovarian steroids are crucial for the development of female secondary sexual characteristics, the regulation of follicle development, driving the menstrual (in humans) and oestrous (in mice) cycles as well as supporting the maintenance of the reproductive tract, pregnancy and lactation. The secretion of the three main sex steroids; androgens, oestrogens and progesterone, is carried out by multiple ovarian cell types. Theca cells are the principal source of androgens, such as testosterone, while the production of oestrogen takes place primarily within the GCs of the developing follicle, through the aromatisation of androgens (McNatty et al., 1979a). Following ovulation, luteinised cells within the corpus luteum (CL) are the primary source of progesterone production (Smith et al., 1975).
1.2 Assembly of the primordial follicle pool

All follicles arise from primordial germ cells (PGCs), the embryonic progenitors of gametes. PGCs are first identifiable during early embryonic development in the yolk sac, before they migrate to the gonadal ridge and expand rapidly by mitosis (Anderson et al., 2000). The PGCs in the gonadal ridge form clusters of germ cell nests (Gomperts et al., 1994, Pepling and Spradling, 2001) and undergo differentiation, ceasing mitosis and entering the first meiotic division, whereupon they are now referred to as oocytes. However, their progress through meiosis I is arrested at the diplotene stage, where they are held in a quiescent state indefinitely or until ovulated, remaining functional for up to 50 years in the human ovary. The oocyte pool undergoes a wave of apoptosis during nest breakdown and the surviving oocytes become surrounded by invading squamous pre-granulosa cells to form primordial follicles (Pepling and Spradling, 2001). Two waves of pre-granulosa cell invasion have been described, first encapsulating oocytes within the ovarian medulla, and a second wave populating cortical oocytes (Mork et al., 2012). The primordial follicle pool is established around the time of birth in rodents (Mork et al., 2012) and during gestation in humans (Baker, 1963).

The abundance of fertilisable oocytes in the ovary has long been considered limited to the finite pool of primordial follicles established before or shortly after birth. However, some controversial studies have reported the existence of ovarian stem cells within the ovarian surface epithelium detected weeks to months after birth in the mouse ovary (Johnson et al., 2004). The reported discovery of mitotically active germ cells within the ovary has been proposed to contribute to a continually replenishing follicular pool. However, these findings have proved divisive, and a recent publication by Zarate-Garcia et al., has disputed the validity of using fluorescence activated cell sorting (FACS) of live cells and an antibody against the germ cell marker DDX4 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4) to isolate these cells (Zarate-Garcia et al., 2016). The authors reported that DDX4 expression was cytoplasmic and not expressed on the cell surface of the mouse oocyte, and the isolated cells were in fact neither DDX4-positive nor did they express germline specific markers upon isolation. Whether or not ovarian stem cells exist and actively contribute to the germ cell pool remains to be proven.
1.3 Follicle development

The development of a quiescent primordial follicle into a large pre-ovulatory follicle primed for ovulation can be divided into two phases; the gonadotropin-independent phase and the gonadotropin-dependent phase (Figure 1.1).

Figure 1.1 Follicle development in the mammalian ovary.

Upon activation dormant primordial follicles develop through the primary, secondary and multi-layered stages. These stages are collectively called preantral follicle development. Although not dependent on gonadotropin signalling, preantral follicles become increasingly responsive to gonadotropins as they grow. Formation of the antral cavity marks the transition to the gonadotropin dependent stage. Few follicles progress further to reach the preovulatory stage and eventually oocytes are released for fertilisation through ovulation. Redrawn and adapted from Kate Hardy.

From birth onwards a small number of follicles are steadily activated to start growing, resulting in the progressive reduction of the quiescent primordial follicle pool. This activation of follicle growth is characterised by oocyte growth, GC proliferation and the transformation of GC morphology from flattened to cuboidal shaped cells (Lintern-Moore and Moore, 1979). Following activation, the oocyte secretes glycoproteins which condense to form an acellular glycoprotein coat called the zona pellucida (Bleil and Wassarman, 1980). Follicles possessing both flattened and cuboidal GCs are called transitional follicles, while the appearance of a complete single layer of cuboidal GCs defines the transition into a primary follicle. As the cuboidal GCs continue to proliferate,
the cells become columnar and polarised, rotating the mitotic axis perpendicular to the basal lamina and begin dividing inwards to form second and subsequent layers (Da Silva-Buttkus et al., 2008). The follicle is now termed a secondary follicle and starts to recruit stromal theca cells on the outward facing aspect of the basal lamina (Young and McNeilly, 2010). Progression up this stage, termed preantral follicle development, takes places in a largely avascular environment and does not require systemic gonadotropin signalling, relying predominantly on local intercellular communication.

The appearance of a fluid filled antral cavity marks the transition to the gonadotropin-dependent phase of development. The progression of antral follicles is reliant on the extraovarian gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), as demonstrated through the development of FSH-deficient female mice which are infertile due to a block in follicle development prior to antrum formation (Kumar et al., 1997). Multiple fluid-filled spaces coalesce to form a single antral cavity which increases in volume as the follicle develops. Antrum formation separates GCs into two functionally distinct populations, mural GCs at the periphery of the follicle, critical for steroidogenesis, and cumulus GCs which surround the oocyte, supporting its growth and developmental competence. The vast majority of follicles in the growing pool will undergo atretic degradation and only a few antral follicles will be selected in each cycle to develop further to the preovulatory stage (Hirshfield, 1991, McGee and Hsueh, 2000). As follicles grow they produce more oestradiol that negatively feeds back to the pituitary, decreasing serum FSH levels and increasing LH production. From a growing cohort dominant follicles are selected, likely those which respond best to falling FSH levels (Son et al., 2011). The selected preovulatory follicles express high levels of LH/human chorionic gonadotropin receptors (LHCGR) within the mural GCs (Peng et al., 1991), and an ovulatory surge in LH signalling results in oocyte meiotic resumption, cumulus expansion, follicle rupture and ultimately the formation of the CL.

1.4 Primordial follicle activation

Initial recruitment of primordial follicles occurs in the medullary region of the ovary shortly after the formation of the primordial follicle pool (Mork et al., 2012). This first wave of activation has been long been considered not to contribute to fertility (Hirshfield, 1992), as the only destination for activated follicles prior to puberty is
atresia. These initial activated follicles are believed to also be the first oocytes to form primordial follicles and enter meiotic arrest during embryonic gonad development, called the ‘production-line’ hypothesis (Henderson and Edwards, 1968). Oocytes within the cortical region of the ovary that undergo slower recruitment of pre-granulosa cells replace the initial wave of primordial follicles and activate later, providing fertility until the end of reproductive life (Hirshfield, 1991, Zheng et al., 2014).

Activation of primordial follicles consists of three morphological transformations; oocyte growth, GC proliferation and GC cuboidalisation (Lintern-Moore and Moore, 1979), however the developmental order of these changes remains unclear. The question of whether the transformation of GCs to a cuboidal shape is a pre-requisite for entry into the cell cycle, or if the shape change is a passive result of increased cell numbers remains unanswered. Studies in mouse ovaries, found significantly higher rates of proliferation, as demonstrated through Ki67 labelling, in cuboidal GCs of activated follicles rather than flat GCs of primordial follicles (Da Silva-Buttkus et al., 2008). However, flat GCs were able to proliferate at a very low level demonstrating that cuboidalisation was not an absolute requirement for proliferation. Additionally, other studies have shown an increase in GC number proceeding the appearance of cuboidal morphology (Lintern-Moore and Moore, 1979), and reported the appearance of proliferation marker, proliferation cell nuclear antigen (PCNA) before GC morphological changes (Oktay et al., 1995). These results suggest proliferation occurs prior to GC shape change. An increase in GC number before the initiation of oocyte growth was first described by Lintern-Moore and Moore in 1979. The authors also showed that oocytes grew more rapidly when surrounded by multiple cuboidal GCs. Additionally, oocytes that have not yet initiated growth can possess cuboidal GCs (Lintern-Moore and Moore, 1979, Da Silva-Buttkus et al., 2008), suggesting the oocyte growth is subsequent to GC proliferation. However, it remains unclear how the oocyte and GCs interact and signal between one another to co-ordinate this transformation.

The initiation of the morphological transformation of GCs from a flattened to cuboidal shape is likely linked to changes in intercellular junctions and the cytoskeleton. Immunolocalisation of the key junctions present between GCs revealed the appearance of both gap junctions and adherens junctions between activated neighbouring GCs,
which appeared to ‘zip up’ as shown through electron microscope imaging (Mora et al., 2012). Specifically, immunofluorescence localisation showed N-cadherin and nectin-2 appearing first at the transitional stage between the wedge-shaped GCs suggesting their involvement in regulating GC shape change (Mora et al., 2012) (Figure 1.2).

**Figure 1.2 N-Cadherin immunolocalisation during follicle activation.**

*N-Cadherin protein (red) first appears between cuboidalising GCs of the transitional follicle, and persist between the GCs of growing preantral follicles. White dotted line indicates basal lamina. GC, granulosa cells; Oo, oocyte; p, primordial follicle; t, transitional follicle; ML, multi-layered follicle. Nuclei are labelled with DAPI (blue). Scale bars = 20 μm. Images courtesy of Jocelyn Mora.*

### 1.4.1 Regulation of primordial follicle activation

The maintenance of primordial follicle dormancy until controlled activation is critical in fertility preservation. It remains unclear, however, if activation is under the control of a stimulatory signal or release of an inhibitory one. In 1996, Wandji et al. proposed that the dormant primordial follicles pool may be under constant inhibitory influences of systemic and/or local origin (Wandji et al., 1996b), and there is increasing evidence that an inhibitory signal originates within the ovary. It has long been established that the number of growing follicles is inversely correlated to the size of the dormant primordial pool (Kraráp et al., 1969). Additionally, if the size of the quiescent follicle pool is severely decreased by *in utero* administration of busalphan to rats, the rate at which the remaining primordial follicles enter the growing pool dramatically increases, resulting
in premature exhaustion of the follicle stockpile (Hirshfield, 1994). Evidence from mathematical modelling of the mouse ovary found that primordial follicles were significantly less likely to initiate growth if they had 1 or more dormant primordial follicles within close proximity (<10 μm) (Da Silva-Buttkus et al., 2009). Taken together, these results indicate that the inhibitory signal may originate from primordial follicles themselves. In vitro cultures have also contributed to the evidence for an inhibitory signal. Whole ovaries isolated from newborn mice and cultured in serum-containing medium show spontaneous activation of primordial follicles in the medullary region of the ovary (Eppig and O’Brien, 1996). Similar results have been obtained from cultures of human ovarian cortical tissue (Telfer et al., 2008) in serum free medium, indicating the presence of an inhibitory signal in vivo, but it is uncertain if the signal has a systemic or local origin. Alternatively, these culture studies could suggest in vitro factors may also play a role stimulating activation.

The regulatory pathways controlling primordial follicle activation are far from defined, however some candidates of ovarian origin have been identified, reviewed by Edson (Edson et al., 2009). The next section contains a brief review of the complex intra-ovarian regulatory factors that are hypothesised to control primordial follicle activation (Figure 1.3), with a focus on local cytokine and growth factors, particularly members of the transforming growth factor-beta (TGF-β) superfamily, reviewed by Knight and Glister (Knight and Glister, 2006).

1.4.1.1 Local ovarian cytokine and growth factors

Ovarian neonatal in vitro cultures have identified a number of local ovarian cytokine and growth factors which enhance the rate of primordial follicle activation. Addition of leukaemia inhibitory factor (LIF) to d4 rat ovary cultures increased the proportion of growing follicles, and anti-LIF neutralizing antibody reduced spontaneous activation (Nilsson et al., 2002). Similarly, platelet-derived growth factor (PDGF) (Nilsson et al., 2006), present in the oocyte, and keratinocyte growth factor (KGF) (Kezele et al., 2005a) when added to culture significantly increased the percentage of activated follicles. Treatment with basic fibroblast growth factor (bFGF), produced by the oocyte, promoted the percentage of follicles undergoing the primordial to primary follicle transition (Nilsson et al., 2001) in cultured neonatal rat ovary.
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Figure 1.3 The complex network of primordial follicle activation regulators.
Ovarian cytokine and growth factor signalling from the stromal cells, neighbouring growing follicles and the primordial follicle itself, both promote and inhibit primordial to primary follicle transition. Blue arrows represent promoters of activation and red arrows represent inhibitors of follicle activation. AMH, Anti-Müllerian hormone; bFGF, Basic fibroblast growth factor; BMP4/7, Bone morphogenetic protein 4/7; FoxL2, Forkhead box L2; GDF9, Growth differentiation factor 9; KGF, Keratinocyte growth factor; KL, Kit ligand; LIF, Leukaemia inhibitory factor; PDGF, Platelet-derived growth factor; PI3K, Phosphatidylinositol 3-kinase; PTEN, Phosphatase and tensin homolog; TGFβ2, Transforming growth factor-beta. Image redrawn and adapted from (McLaughlin and McIver, 2009).

1.4.1.2 TGF-β superfamily
The TGF-β superfamily has been heavily implicated in the regulation of primordial follicle activation. Anti-Müllerian hormone (AMH) is expressed within the GCs of growing follicles (Durlinger et al., 2002b, Weenen et al., 2004), peaking in large preantral follicles. Whole mouse ovaries cultured with AMH showed a significant reduction in the proportion of follicles recruited into the growing pool (Durlinger et al.,
2002a), and similar results have been found in cultured human ovarian tissue (Carlsson et al., 2006). Furthermore, AMH deficient female mice display premature depletion of primordial follicles by 4 months of age (Durlinger et al., 1999). These studies indicate that AMH is an important negative regulator of primordial follicle activation produced by growing follicles. AMH type II receptor (AMHRII) is expressed in GCs of primary, preantral and small antral follicles, but interestingly not primordial follicles (Baarends et al., 1995), suggesting an indirect mechanism by which AMH suppresses follicle activation. Additionally, recent work produced from within our group has highlighted another TGF-β family ligand as a negative regulator of activation, TGFβ2, produced in the oocyte, inhibited primordial follicle development when added to mouse neonatal ovary cultures (Oliver, 2017). In contrast, other members of the TGF-β superfamily such as bone morphogenetic proteins, BMP4 (Nilsson and Skinner, 2003) and BMP7 (Lee et al., 2001) have both been shown to stimulate the transition of follicles from the primordial stage to the pool of growing follicles in vitro. Growth differentiation factor-9 (GDF9) is expressed specifically within the oocyte of follicles (McGrath et al., 1995). Although the addition of GDF9 had no effect on follicle activation in cultured neonatal rat ovaries (Nilsson and Skinner, 2002), GDF9 did increase rates of primordial follicle activation when added to cultured human ovarian tissues and may have an indirect role on activation (Hreinsson et al., 2002). SMADs are intracellular transcription factors activated downstream of the TGFβ ligands. Smad2/3 have been localised to primordial, transitional and primary follicles, while Smad1/5/8 are principally expressed in larger multi-layered follicles (Fenwick et al., 2013). Recent observations from within our group showed that expression of nuclear Smad2/3 and the proliferation marker Ki67 was mutually exclusive in the majority of nuclei in early stage mouse follicles, suggesting that activity of Smad2/3 is associated with the maintenance of GC dormancy in the primordial follicle (Hardy et al. unpublished observations), further implicating upstream TGF-β signalling in follicle activation.

1.4.1.3 Intracellular signalling pathways

Studies of oocyte intracellular signalling pathways have highlighted the phosphatidylinositol 3-kinase (PI3K) pathway as vital for primordial follicle survival and development (Reddy et al., 2005, Liu et al., 2006). An important ligand and receptor pair known to activate PI3K pathway, are the receptor tyrosine kinase Kit (c-Kit)
expressed at the oocyte surface and its ligand, Kit ligand (KL) expressed by GCs. Cultured neonatal rat ovaries display a dramatic increase in primordial follicle activation with the addition of KL, the effect of which is completely blocked with the addition of the c-Kit neutralising antibody ACK2 (Parrott and Skinner, 1999). Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene and a negative regulator of PI3K. Oocyte-specific deletion of Pten in mice causes over-activation of primordial follicles that results in the early depletion of the entire follicle pool (Reddy et al., 2008). These studies demonstrate that the PI3K/PTEN pathway in the oocyte plays an important role in governing follicular activation. Additionally, transcription factors activated downstream of PI3K/AKT, such as Forkhead box O3 (Foxo3a) expressed in the nuclei of oocytes in the primordial follicle have been implicated in activation. Foxo3a deficient mice display total depletion of follicles by 15 weeks of age due to over-activation of the primordial follicle pool (Castrillon et al., 2003).

Intracellular transcription factor forkhead box L2 (Foxl2) is expressed in pre-granulosa cells and also regulates activation. Foxl2−/− knockout mice do not develop secondary follicles because GCs do not undergo the cuboidal transition, indicating that Foxl2 is important for the GC transformation. The majority of oocytes activate prematurely and undergo atresia resulting in depletion of the follicle pool (Schmidt et al., 2004).

These studies taken together have demonstrated that primordial follicle activation is controlled by complex signalling pathways between oocytes, GCs and stromal tissue. The convergence of multiple activator and repressor pathways is likely necessary for tightly controlled activation.

1.4.2 Pathologies associated with dysregulation of primordial follicle activation

Controlled activation of the primordial follicle pool is essential for the maintenance of fertility, as over-activation can lead to early depletion of primordial follicles and premature aging of the ovary. In humans, premature ovarian insufficiency (POI) is defined as the loss of normal function of the ovaries before age 40, and affects approximately 1% of women (Beck-Peccoz and Persani, 2006). Despite increasing research into genetic irregularities underlying POI, the vast majority of cases are
idiopathic (Goswami and Conway, 2005). The aetiology of POI is unclear, but may be associated with a deficiency during the primordial follicle formation, increased atresia of primordial follicles or accelerated activation of follicles. Elucidation of the mechanisms underlying primordial follicle activation may contribute to our understanding of the aetiology of POI and help to direct research toward potential therapeutic targets.

1.5 Preantral follicle development

Following activation, the cuboidal GCs continue to proliferate. Once a full layer of cuboidal GCs surrounds the oocyte, the GCs begin to divide inward to form subsequent layers. The columnar GCs become polarised and tightly packed, resulting in the rotation of the mitotic axis so that it is perpendicular to the oocyte (Da Silva-Buttkus et al., 2008). During preantral follicle development, oocyte growth is significant, increasing up to 300 times in size during a 2- to 3-week growth phase in the mouse (Adhikari and Liu, 2009, Wassarman and Albertini, 1994). This growth phase is accompanied by a considerable increase in RNA content and protein synthesis by the oocyte. The growing oocyte secretes glycoproteins which assemble into long fibrils and form the zona pellucida (Bleil and Wassarman, 1980). The zona pellucida is penetrated by trans-zonal cytoplasmic processes that contact the oocyte, allowing direct communication between the oocytes and GCs. During preantral development the follicle recruits stromal theca cells to the basal lamina. The acquisition of a theca layer is an important developmental step and is associated with increased follicular growth as well as an increased steroidogenic response to gonadotropins (Wandji et al., 1996b). The theca layer supplies structural support and increases blood supply containing important systemic regulators of follicle development (Young and McNeilly, 2010). Additionally, the theca cells are the primary source of androgen synthesis in the developing follicle, increasing availability of substrate for oestrogen biosynthesis and directly affecting follicle growth, described in detail in Section 1.6. Theca cells are thought to originate from either cortical or medullary stromal cells. Work undertaken in the bovine ovary has shown that co-culture of GCs from small antral follicles with ovarian cortical stromal cells can promote differentiation of stromal cells into theca cells and increase their LH responsiveness (Orisaka et al., 2006). This suggests that interaction between GCs and cortical stromal cells is important in the formation of the theca cell layer.
The progression of follicle development has been suggested to be accompanied by a partial epithelial-mesenchymal transition (EMT) following primordial follicle activation (Mora et al., 2012). Mora et al. demonstrated that surprisingly, GCs from developing mouse follicles did not express epithelial markers such as E-cadherin, Cytokeratin-8 and Zonula occludens-1, but did express vimentin, suggesting a more mesenchymal phenotype. It has been proposed previously that GCs undergo EMT upon ovulation, losing their polarity to become luteal cells within the CL (Irving-Rodgers et al., 2004). Mora et al., postulated that GCs undergo partial EMT following activation that completes upon ovulation. However, the signalling mechanisms driving this EMT remain elusive.

1.5.1 Regulation of preantral follicle growth

Growth and survival of preantral follicles within the ovary must be tightly regulated in order to ensure fertility. The precise signalling pathways controlling these early developmental stages remain poorly understood, however are thought to be controlled by local growth factors as well as ovarian steroids and pituitary produced gonadotropins, reviewed by Edson (Edson et al., 2009). The next section will briefly review the known systemic and local regulators of preantral follicle development (Figure 1.4).

1.5.1.1 Systemic factors

The preantral stages of follicle growth have long been recognised as gonadotropin-independent, however these small follicles appear to be gonadotropin responsive. The FSH receptor (FSHR) is expressed from the early primary stage of follicle development (Oktay et al., 1997), and in vitro studies have shown that preantral follicles respond to FSH when added to culture. Recent work from within our group has shown that mouse preantral follicles grow significantly larger when cultured with FSH and become progressively responsive with increasing size (Hardy et al., 2017). Additionally, in vivo data has shown that reduction of gonadotropin signalling through either hypophysectomy or GnRH antagonist treatment in prepubertal female rats, resulted in decreased ovarian weight associated with a reduced number of developing preantral (McGee et al., 1997), suggesting FSH is important for preantral follicle survival. However, FSHR-null mice can still develop preantral follicles, showing that gonadotropin signalling is not a requirement for these early stages (Abel et al., 2000).
Figure 1.4 Regulation of preantral follicle growth and survival.
Systemic, as well as oocyte, granulosa, theca and stomal cell derived factors form a complex network to regulate the development and growth of the preantral follicle. A balance exists between negative (red arrows) and positive (blue arrows) regulators. AMH, Anti-Müllerian hormone; bFGF, Basic fibroblast growth factor; BMP4/7/15, Bone morphogenetic protein 4/7/15; EGF, Epidermal growth factor; FGF-10, Fibroblast growth factor-10; FSH, Follicle stimulating hormone; GDF9, Growth differentiation factor 9; IGF-1, Insulin-like growth factor 1; KGF, Keratinocyte growth factor; KL, Kit ligand; TGFβ2, Transforming growth factor-beta; VEGF, Vascular endothelial growth factor.

1.5.1.2 Oocyte-derived factors
Preantral follicles located in the cortex of the ovary develop in an environment that is largely avascular, relying predominately on local autocrine and paracrine intra-ovarian regulators. Although the oocyte relies on the supporting somatic cells, there is also evidence that oocyte-derived factors play a central role in regulating the progression of preantral follicle development. Studies performed by Vanderhyden et al., showed that oocyctectomised preantral follicles (microsurgical procedure that removes the oocyte while retaining the three-dimensional structure of the follicle), displayed reduced GC proliferation (Vanderhyden et al., 1992), and altered steroidogenesis (Vanderhyden and Macdonald, 1998), the effect on growth could be reversed by exposure to oocyte
conditioned medium. The authors postulated that the rate of follicular development is orchestrated by mechanisms intrinsic to the oocyte (Eppig et al., 2002).

The principal oocyte-derived factor identified as critical for preantral follicle development is GDF9. GDF9 is present though preantral follicle growth, first appearing within the oocytes of primary follicles (McGrath et al., 1995) and has been shown to stimulate preantral follicle growth when added to culture (Hayashi et al., 1999). Furthermore, GDF9-deficient female mice display a block in follicle development at the primary stage resulting in infertility (Dong et al., 1996). The GDF9 null mice showed reduced GC proliferation, and follicles failed to form a theca layer (Elvin et al., 1999), demonstrating that oocyte produced GDF9 is required for GC multi-layering and theca recruitment. Other oocyte derived factors, such as BMP15, although having been shown to stimulate GC proliferation in culture (Otsuka et al., 2000), only become essential in later follicle development, as BMP15 null mice are sub fertile displaying decreased ovulation and fertilisation rates (Yan et al., 2001). Recent work from within our group has shown that TGFβ2 ligand produced in the oocyte can also stimulate the growth of isolated mouse preantral follicles in culture (Elizabeth Oliver, PhD Thesis 2017).

1.5.1.3 Somatic cell-derived factors
A large number of factors secreted by granulosa, theca and stromal cells have been implicated in the complex intraovarian control mechanisms of preantral follicle development. KL produced by the preantral GCs (Joyce et al., 2000) signals through its receptor c-KIT on the oocyte surface and theca cells, with ligand expression peaking in larger preantral follicles in the mouse (Manova et al., 1993). In vitro studies have shown that KL can promote oocyte growth (Packer et al., 1994) and GC proliferation (Reynaud et al., 2000, Otsuka and Shimasaki, 2002) in preantral follicles. Furthermore, mutations of c-Kit in mice result in defects at the primary stage of development causing infertility (Huang et al., 1993).

Ligands within the TGF-β family have again been highlighted as important regulators of preantral follicle growth. Intraovarian activins produced by the GCs promote preantral follicle growth, increases FSHR expression and promote oestrogen synthesis, reviewed by Knight and Glister (Knight and Glister, 2001). Particularly the isoform activin A has
been shown to signal in an autocrine manner to stimulate GC proliferation (Yokota et al., 1997, Liu et al., 1999). TGF-β ligands produced in the theca such as BMP-4 and BMP-7 signal through BMP receptor types IA, IB, and II in GCs and oocytes to modulate oestradiol synthesis and inhibit progesterone synthesis (Shimasaki et al., 1999). BMP-7 has been shown through in vivo models to promote follicle growth beyond the primary stage of development (Lee et al., 2001). Additionally, AMH, which plays a critical role as a negative regulator of primordial follicle activation, also has a role to play in preantral follicle growth. AMH in culture has been shown to inhibit FSH stimulated preantral follicle growth in the mouse (Durlinger et al., 2001).

In vitro cultures have highlighted various growth factors as having stimulatory effects on preantral follicle growth. Expression of insulin-like growth factor 1 (IGF-1) and IGF1 Receptor (IGF1R) are high during preantral follicle development (Levy et al., 1992). In vitro cultures of mouse preantral follicles showed that IGF-1 added in combination with FSH produced a synergistic effect on follicle growth (Liu et al., 1998). However, the development of IGF-1 null mice, showed that although later stage follicles fail to respond to gonadotropins, they develop normally to the late preantral stage (Baker et al., 1996), suggesting IGF-1 signalling is not essential for follicular development until the early antral stage. Other growth factors include vascular endothelial growth factor (VEGF) which has been shown to stimulate preantral follicle growth in a time- and dose-dependent manner in the rat ovary (Danforth et al., 2003). Additionally, KGF has been detected in theca cells and may signal in a paracrine manner through KGF receptors (KGFR) detected in GCs of preantral follicles (Parrott and Skinner, 1998). KGF when added to culture promotes the survival, growth and differentiation of preantral follicles in the rat ovary (McGee et al., 1999). bFGF localised to the oocyte of primordial and primary follicles (Nilsson et al., 2001), when added to culture increases the incorporation of [3H]thymidine into isolated small bovine preantral follicles (Nuttinck et al., 1996, Wandji et al., 1996a), and promotes growth of goat preantral follicles (Matos et al., 2007). Similarly, work in the goat ovary was shown that fibroblast growth factor-10 (FGF-10) (Chaves et al., 2010) promotes growth of developing follicles. The physiological roles of most of these growth factor ligands in preantral follicle growth in vivo is not clear, as many of these studies employ in vitro culture systems and few ovary-specific growth factor knockout mice have been generated.
1.5.1.4 Gap junctions

The basal lamina acts as a barrier between the GCs and vascularised theca cells, isolating GCs from the ovarian blood supply. An extensive network of gap junctions exists allowing communication and transportation of ions, metabolites, and small molecules between GCs and GCs and the oocyte. The gap junction protein connexin 37 (Cx37) is expressed at the oocyte-GC interface at all stages of follicle development peaking in the preantral stage (Teilmann, 2005). Mice deficient in Cx37 display defective late preantral follicle development due to impaired oocyte-granulosa communication (Simon et al., 1997). Connexin 43 (Cx43) is expressed between the GCs of all follicular stages, in particular in the transition from pre-antral to the antral stage (Teilmann, 2005). Similarly, mice with a null mutation in the Gja1 gene encoding Cx43 display arrested development at the primary stage (Juneja et al., 1999). Regulation of these gap junctions is essential during preantral follicle development.

Ovarian steroids, particularly androgens, also have significant effects on preantral follicle development. The role of androgens in the preantral follicles is covered in detail in Section 1.6.

1.5 Epidermal growth factor family

The epidermal growth factors (EGF) and their receptors play an essential role during ovulation, mediating the response to the LH surge, however they have received little attention for their role during primordial follicle activation and preantral follicle growth. The expression of the EGF-like ligands and their receptors, collectively known as the ErbBs, is well characterised in the preovulatory follicle however our group has recently produced evidence that they may also be present and active much earlier in follicle development.

The EGF-like ligands and their receptors are implicated in a wide range of biological responses, including cell division, migration, adhesion, differentiation and apoptosis. The diversity of responses is mediated by a complex signalling network, which involves a variety of EGF-like ligands which can stimulate the formation of ErbB dimers in various combinations at the cell surface to induce a complex network of intracellular downstream signalling pathways, reviewed by Yarden (Yarden and Sliwkowski, 2001).
1.5.1 The epidermal growth factor receptors (ErbBs)

The ErbB family of structurally related receptor tyrosine kinases (RTKs) consist of four receptors; ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4. Each receptor comprises an extracellular domain to facilitate ligand binding, an α-helical hydrophobic transmembrane segment and an intracellular protein tyrosine kinase and C-terminal tail domain (Olayioye et al., 2000) (Figure 1.5).

![Figure 1.5 The four ErbB family members.](image)

Each receptor consists of an extracellular domain, responsible for ligand binding and dimerisation, a transmembrane domain, and an intracellular cytoplasmic domain, responsible for tyrosine kinase activity. ErbB2 contains no ligand-binding cleft and exists in a constitutively active formation with its dimerisation loop extended (yellow dot). EGFR, ErbB3 and ErbB4 exist in a tethered confirmation with the dimerisation loop masked until ligand binding causes a conformational change, exposing the loop. The ErbB3 cytoplasmic domain has little or no kinase activity. Redrawn and adapted from Kate Hardy.

Dimerisation between the ErbB receptors can occur between two different receptors (heterodimerisation) or between two of the same receptors (homodimerisation). The ErbB receptors differ in their dimerisation abilities. Upon ligand binding EGFR and
ErbB4 have the capacity to form both homo- and hetero-dimers (Yarden and Sliwkowski, 2001). In contrast, ErbB2 is an ‘orphan receptor’ and has no known binding ligand (Klapper et al., 1999), however its conformation resembles a ligand-activated state and is poised to form heterodimers with all the other ErbB subtypes to signal (Cho et al., 2003, Garrett et al., 2003). ErbB3 can be successfully bound by ligands but has has little or no kinase activity (Guy et al., 1994) and although ErbB3 homodimers can form they possess little autophosphorylation ability (Shi et al., 2010). However, the weak ErbB3 kinase domain can still serve as an activator and signals primarily through heterodimerisation.

Hierarchical relationships of homodimer and heterodimer formation have been characterised by introducing ErbB receptor combinations to ErbB naïve cell populations and by the use of specific ErbB blocking antibodies (Tzahar et al., 1996, GrausPorta et al., 1997). These studies revealed a strict hierarchy of inter-receptor interactions. Although ErbB2 does not bind ligands it is the preferred heterodimerisation partner of all other ErbB receptors, while ErbB2 itself favours interaction with ErbB3 (Tzahar et al., 1996, Holbro et al., 2003). Interestingly, despite their distinct deficiencies, ErbB2 and ErbB3 together form the most potent signalling pair in terms of mitogenic response (Holbro et al., 2003). Heterodimers containing ErbB2 are the most potent complexes (PinkasKramarski et al., 1996), as ErbB2 can potentiate and prolong signal transduction pathways, enhance ligand affinity, relax ligand specificity and alter receptor trafficking (GrausPorta et al., 1997).

The crystallisation of unbound and ligand-bound ErbBs has helped to elucidate the physical interactions of the receptors upon dimerisation. The extracellular region of the ErbB receptors contain four distinct domains. Domains I and III are related to the leucine-rich repeat superfamily and adopt the right-handed β helix shape. Domains II and IV are both cysteine-rich domains containing small disulfide-bonded modules and adopt laminin-like folds (Burgess et al., 2003). The extracellular domains of EGFR, ErbB3 and ErbB4 exist in two confirmations, either as tethered, auto-inhibited form or upon ligand binding in an untethered form (Yarden and Pines, 2012, Lemmon, 2009). In the absence of ligand, the ErbB extracellular region adopts a tethered configuration, in which the dimerisation arm located at domain II is buried by intramolecular...
interactions with domain IV, which auto-inhibits dimerisation (Ferguson et al., 2003). Upon exposure to ligands, each ligand binds to one receptor molecule (Ogiso et al., 2002) and makes contact with domains I and III drawing them together, resulting in a conformational change, extending the receptor and exposing the dimerisation interfaces in domain II. The exposed dimerisation arm is responsible for the formation of both homo- and hetero- dimers (Greenfield et al., 1989). In contrast, the subtype ErbB2 exists in a constitutively activated conformation similar to that of ligand-bound EGFR, with the receptor extended and its dimerisation arm constitutively exposed (Garrett et al., 2003). Dimerisation of the ErbBs is mediated entirely by intermolecular contacts between the receptors with no contribution from the bound ligand at the dimer interface (Lemmon, 2009). This receptor mediated dimerisation makes ErbBs unique among other RTKs which classically use the ligand as a bridge between receptors. Dimerisation of receptors is followed by rearrangements in the transmembrane and juxtamembrane domains. Kinase activation leads to ErbB receptor auto- and trans- phosphorylation, which initiates a cascade of intracellular signalling.

1.5.2 Downstream signalling of the ErbBs
The four ErbB receptors each have distinct locations and numbers of tyrosine phosphorylation sites on their C-terminal tail (Figure 1.6). Which sites on the C-terminal tail become phosphorylated depends on the stimulatory ligand that binds and the composition of the dimer formed. Activated receptors auto- and trans- phosphorylate each other on multiple tyrosine residues, which serve as docking sites for the Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains of downstream adaptor proteins. Dependent on which tyrosine residues are phosphorylated, particular phosphotyrosine binding proteins will attach to the receptors to propagate the intracellular signal (Baselga and Swain, 2009).

A vast array of intracellular signalling cascades have been elucidated downstream of the ErbB receptors, including the mitogen-activated protein kinase (MAPK), PI3K, phosphoinositide phospholipase C-protein kinase C (PLCγ-PKC), c-Jun N-terminal kinases (JNK), and Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathways (Wee and Wang, 2017). Many of these pathways are interlinked, resulting in an entire network of signalling that is activated downstream of the ErbBs.
Figure 1.6 Downstream signalling pathways of the ErbBs.

EGFR, ErbB2, ErbB3 and ErbB4 possess docking sites for Src homology 2 (Shc), growth factor receptor binding protein (Grb), phosphoinositide phospholipase C (PLCγ) and p85 in varying quantities. Downstream signalling cascades of the ErbBs include protein kinase C (PKC), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT, and janus kinase (JAK) signaling. EGFR dimers can directly activate MAPK, PKC and JAK/STAT cascades and indirectly activate the PI3K/AKT pathway. ErbB3 and ErbB4 can directly activate PI3K/AKT as they possess binding sites for the PI3K regulatory subunit p85. Turquoise receptor represents ErbB dimerisation partner. Redrawn and adapted from (Yarden and Pines, 2012).

1.5.2.1 MAPK signalling cascade

The MAPK signalling pathway is activated downstream of all ErbB dimer combinations (Yarden and Sliwkowski, 2001), and may be the most important in mediating the biological response of the EGFR (Wee and Wang, 2017). In brief, following dimerisation and transphosphorylation of the ErbB receptor, growth factor receptor binding protein 2 (GRB2) binds via its SH2 domain to activated residues on the ErbB cytoplasmic tail. Additionally, another adaptor protein, Src homology and collagen (SHC) is recruited preferentially through its PTB domain. SHC is phosphorylated and becomes a binding
site for GRB2 and the two associate with each other. The two Src homology 3 (SH3) domains of GRB2 bind to proline rich carboxy-terminal tail in son of sevenless 1 (SOS1). SOS1 is a guanine nucleotide exchange factor (GEF) for RAS small guanosine triphosphatase (GTPase), and activates the protein RAS by inducing the exchange of GDP to GTP. Activated RAS then interacts with the Ras-GTP-binding domain (RBD) on RAF-1 to phosphorylate it. Phosphorylated RAF-1 directly activates and phosphorylates mitogen-activated protein kinase kinase (MEK1/2). MEK1/2 is a tyrosine and threonine/serine dual-specificity kinases that activates extracellular signal-regulated protein kinases 1 and 2 (ERK1/2). The activated ERK1/2 can interact with over a hundred substrates to initiate cellular responses in growth, proliferation, differentiation, migration, and inhibition of apoptosis.

1.5.2.2 PI3K signalling cascade
The PI3K (PI3K-AKT-mTOR) is activated by direct coupling with ErbB3 and ErbB4 and activated through indirect mechanisms by EGFR and ErbB2 (Yarden and Sliwkowski, 2001). PI3K is comprised of a regulatory p85 subunit and a catalytic p110 domain that phosphorylates the 3-OH group of membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 is the predominant mediator of PI3K activity, and stimulates the activation and phosphorylation of AKT. The p85 SH2 domain recognises the phosphorylated motifs present in ErbB3 and ErbB4 and binds directly to the receptors. However, EGFR and ErbB2 stimulation of this pathway is mediated by adaptor protein GRB2-associated binder (GAB1), which contains p85 subunit PI3K binding sites. Alternatively, activated RAS can bind and recruit PI3K by the p110 subunit. AKT is a serine/threonine kinase and mediates a response through the activation or deactivation of several downstream proteins, such as mTOR, to impact changes in metabolism, proliferation, cell size, survival and motility.

The downstream cascades activated are dependent on dimer composition. In the case of the most potent ErbB2-ErbB3 dimer combination, the cytoplasmic tail of ERBB3 strongly activates the PI3K survival pathway, whereas ERBB2 signals through the MAPK pathway to stimulate proliferation. The combination of PI3K and MAPK signalling results in a strong mitogenic and anti-apoptotic effect (Yarden and Pines, 2012).
1.5.2.3 Transcriptional changes
These intracellular signalling cascades translate to the nucleus to regulate distinct transcriptional changes. Transcription factors activated downstream of ErbB signalling include FOS, JUN, MYC, SP1, early growth response 1 (EGR1), and GA-binding protein (GABP) (Wee and Wang, 2017). There has been some evidence that upon EGF stimulation ErbBs can also translocate to the nucleus (Lin et al., 2001). Full length EGFR, ErbB2 and ErbB3 have been found in the nucleus of various cancer cell types. The mechanisms underlying EGFR nuclear translocation remain unclear, however, a nuclear localisation signal (NLS) has been detected with the EGFR. Within the nucleus, EGFR has been proposed to alter gene expression through acting as a transcriptional co-activator to stimulate cell cycle progression mediators such as Cyclin D1.

1.5.3 The epidermal growth factor ligands
The EGF family of growth factor ligands include EGF, transforming growth factor-α (TGF-α), heparin-binding EGF (HB-EGF), amphiregulin (Areg), betacellulin (Btc), epiregulin (Ereg) and epigen (Epgn) (Harris et al., 2003). The seven peptides share an EGF-like motif in their extracellular domain which is responsible for binding and activating the ErbBs (Schneider and Wolf, 2009). EGF, TGF-α, Areg and Epgn are specific ligands for the EGFR, while Btc, HB-EGF and Ereg show dual specificity for both EGFR and ErbB4 (Figure 1.7). Additionally another class of ligands, the neuregulins (NRGs), bind both ErbB3 and ErbB4 (Falls, 2003).
EGFR-like ligands are synthesised as membrane bound precursors. The availability of the ligands is mediated by their proteolytic cleavage of the extracellular fragment from the cell membrane. Shedding of ligands is controlled by cleavage enzymes such as matrix metalloproteinases (MMP) and those in the ADAM family of metalloproteinases. In particular, ADAM10 and ADAM17 have been implicated in the ectodomain shedding of six of the seven EGFR ligands (Sahin et al., 2004). Membrane anchored EGF-like ligands, such as HB-EGF and TGF-α are able to activate ErbBs on adjacent cells through a process known as juxtacrine signalling. The cleaved soluble growth factors ligands have the ability to activate receptors further afield on distinct cells as well as neighbouring cells and the cell of origin (Schneider and Wolf, 2009).
Each ligand appears to bind to the ErbB receptors via similar mechanisms, however the binding of the differing ligands has been shown to cause distinct downstream biological activities. The mechanisms underlying the differences induced by each ligand remain unclear, however some theories have been put forward. Ligands have varying affinities for the ErbB receptors and can induce preferred dimer pairs to stimulate a range of downstream signalling pathways. Additionally, it has been proposed that the ligand identity contributes to the fate of the receptor. Following endocytosis, endocytic sorting generally targets EGF, HB-EGF and Btc bound receptors for lysosomal degradation, whereas binding of TGF-α, Areg or Epgn results in receptors largely recycling back to cell membrane (Roepstorff et al., 2009). The differences in endocytic sorting fates may be attributable to varying pH sensitivities of each ligand bound receptor. For example, EGF and TGF-α have been shown to bind to EGFR with comparable affinities at pH 7.4. However, a decrease to pH 6, similar to the pH in early endosomes, results in increased TGF-α disassociation from EGFR, allowing the unbound receptor to be recycled back to cell membrane, whereas EGF dissociation was significantly lower, leading to trafficking toward lysosomal degradation (French et al., 1995). Differential sorting can lead to more potent and prolonged signalling activity of some EGF like ligands, particularly TGF-α, through sustaining EGFR presence at the cell surface (Waterman et al., 1998).

1.5.4 Recycling of ErbBs
A combination of positive and negative feedback loops regulates the output of the ErbB receptors dictating the duration, amplitude and frequency of signals. The regulatory loops fall into two categories, immediate and late (Avraham and Yarden, 2011).

Immediate negative regulation of the active ErbB receptors can occur through receptor tyrosine dephosphorylation. This process is mediated by phosphatase enzymes which remove docking sites for downstream adaptor proteins such as GRB2 and SHC. Additionally, ligand-mediated receptor endocytosis is a major negative feedback mechanism. The ligand-receptor complex is internalised and sorted for either recycling or degradation by lysosomes. For example, activated EGFR recruits GRB2 as well as the E3 ubiquitin ligase CBL, which ubiquitylates EGFR. Ubiquitins on EGFR are then recognized by ubiquitin-binding proteins that promote receptor endocytosis into the early endosomes. The internalised receptors can remain active, signalling intracellularly
from endosomes to prolonging the response. Once in the early endosomes, ligand dissociation promotes de-ubiquitination of the receptor and unoccupied EGFR receptors are rapidly recycled back to the cell membrane. In contrast, ligand-bound receptors are either targeted for degradation by lysosomes or recycled back more slowly. The composition of the dimer pair can mediate recycling fate. EGFR heterodimers containing ErbB2 often evade lysosomal degradation, and instead are preferentially recycled back to the cell membrane, resulting in increased signal duration (Waterman et al., 1998). ErbB2, therefore, prevents negative regulation of activated EGFR and leads to increased concentrations of available ErbBs at the cell surface.

Delayed feedback loops involve newly synthesised proteins induced by EGF-like ligand stimulation. ErbB stimulation can alter the downstream transcription and translation of EGF-like ligands and ErbB receptors to mediate signalling long term. ErbB stimulation can also increase expression of signal attenuators such as LRIG1, which associates with all ErbB proteins to increase CBL recruitment and accelerate their degradation, or receptor-associated late transducer (RALT) which binds directly to tyrosine kinase domain of ErbBs and inhibits phosphorylation (Wieduwilt and Moasser, 2008).

1.5.5 Links to development and cancer

The ErbB family of receptors are essential for vertebrate embryogenesis, and null mutations of any of the ErbB genes are embryonically or perinatally lethal. The EGFR is implicated in a wide range of cellular activities, as shown by EGFR null mice that show abnormalities in skin, kidney, brain, liver, and gastrointestinal tract (Threadgill et al., 1995). Mice carrying an ErbB2 null allele display lethal defects in neural and cardiac development, and mutant embryos do not survive past embryonic day 11 (Lee et al., 1995). Additionally, targeted mutations in ErbB3 results in mice that display defective cardiac formation and cerebella development (Erickson et al., 1997), and mice lacking ErbB4 show it is essential for cardiac muscle differentiation and axon guidance in the central nervous system (Gassmann et al., 1995). Transgenic and knock-out mice for the various ErbB ligands display much less severe phenotypes, demonstrating a degree of redundancy between the ligands (Wong, 2003).
Perturbed ErbB signalling can result in unrestrained cell growth and survival, and has been found to underlie numerous cancers. Over expression of wild type ErbB receptors has been detected in a wide range of tumours obtained from cancer patients. Gene amplification of ErbB2 resulting in overexpression of the receptor is found in approximately 30 % of breast cancers, and is associated with overall survival and time to relapse in patients (Slamon et al., 1987). Amplification of EGFR has been found in at least 10 cancer types and overexpression is a strong prognostic indicator in ovarian, cervical, bladder and oesophageal cancers (Nicholson et al., 2001). Additionally, mutations in the ErbB receptors have been identified in cancers of the breast, lung, colon, stomach, pancreatic, ovary, brain, prostate, and kidney (Hynes and Lane, 2005, Wee and Wang, 2017). Activating tyrosine kinase domain mutations have been identified in EGFR but also in ErbB2, as well as in-frame deletions in the extracellular domain of EGFR. These mutations promote constitutive receptor activation, which enhances progression of cancer cell proliferation and survival.

1.5.6 Role of ErbBs in the ovary

Most research to date has focused on the role of ErbBs during preovulatory follicle development, however there is preliminary evidence that the ErbBs may also play a role in the early preantral follicle.

1.5.6.1 Established role of EGF family in ovulation

The role of the EGF-like ligands and ErbBs during ovulation is well characterised (Conti et al., 2006). The ErbB receptor subtype EGFR is essential for normal ovulation (Park et al., 2004), as shown by targeted ablation of EGFR in GCs which results in disrupted oocyte maturation and ovulation in mice (Hsieh et al., 2011). Additionally, mice null for one of the EGF-like ligands display attenuated meiotic resumption, however, these mice display a less severe phenotype than the EGFR null mice, suggesting compensation from the other EGF-like ligands (Kim et al., 2011, Hsieh et al., 2007).
Figure 1.8 The role of ErbB signalling during ovulation.
The luteinizing hormone (LH) surge stimulates the transcription and shedding of EGF-like ligands, amphiregulin (Areg), epiregulin (Ereg) and betacellulin (Btc), in the mural granulosa cells (GCs) which diffuse across the antral cavity to activate EGFR in the cumulus GCs surrounding the oocyte. LH induced EGFR signalling contributes to resumption of meiosis in the oocyte and germinal vesicle breakdown (GVBD) and cumulus expansion during ovulation. Redrawn and adapted from Kate Hardy.

The LH surge stimulates the preovulatory follicle to induce the resumption of meiosis in the oocyte, cumulus cell expansion and GC luteinisation (Figure 1.8). LH receptor is expressed in the mural GCs around the periphery of the follicle, lining the antrum, however is absent from the cumulus GCs surrounding the oocyte and the oocyte itself. This puzzling expression pattern led to the discovery of intermediate signalling factors, the EGF- like ligands, Areg, Ereg and Btc. The addition of EGF ligand to follicle-enclosed oocytes has long been shown to induce oocyte maturation in culture (Dekel and Sherizly, 1985). However, it is the transcription of EGF-like ligands, Areg, Ereg and Btc, which is rapidly and transiently induced by LH in the mural GCs of the preovulatory follicle (Park et al., 2004). Furthermore, the addition of exogenous Ereg, Areg, and Btc to
culture medium mimics some of the actions of LH, inducing resumption of meiosis in rat preovulatory follicles (Ashkenazi et al., 2005). Phosphorylation of EGFR and MAPK has been observed in the cumulus cells of rat preovulatory follicles 30 minutes after LH stimulation, which can be blocked with the addition of an EGFR kinase inhibitor, AG1478, or the addition of neutralizing antibodies against Areg, Ereg or Btc (Panigone et al., 2008). As mentioned previously, the EGF-like ligands are translated as membrane bound precursors, which are released from the cell surface as mature, soluble peptides by proteolytic cleavage. Inhibition of growth factor shedding by the addition of MMP inhibitor GM6001 also blocks the effects of LH on maturation and cumulus expansion in cultured preovulatory follicles (Panigone et al., 2008). Furthermore, it has been reported recently that the ErbB subtype ErbB2 becomes phosphorylated after human chorionic gonadotropin (hCG) stimulation in mouse ovaries (Kim et al., 2011), potentially implicating EGFR-ErbB2 heterodimers in the response to ovulatory LH. These studies have cemented the role of both EGF-like ligands and EGFR in relaying of the LH signal during ovulation.

1.5.6.2 Current evidence for EGF family role in preantral follicle development

To date, research into expression and activity of the ErbBs in the ovary has focused on the pre-ovulatory follicle, and there has been limited investigation into the earlier stages of follicle development. However, few studies have detected ErbB mRNA and protein expression in small preantral and primordial follicles in the hamster (Garnett et al., 2002) and rat (Xu et al., 2009) ovary. Additionally, inhibition of ErbB2, has been shown to abrogate spontaneous activation of rat primordial follicles during culture of whole ovaries (Li-Ping et al., 2010).

Supplementation of culture medium with EGF has been shown to affect survival and development of preantral follicles during in vitro maturation in other model species. Exposure to EGF in vitro increases the size of bovine (Gutierrez et al., 2000), equine (Aguiar et al., 2017), caprine (Silva et al., 2013, Celestino et al., 2011, Silva et al., 2004) and ovine (Santos et al., 2014) preantral follicles, as well as providing beneficial effects on follicular morphology and viability in caprine (Celestino et al., 2009), feline (Fujihara et al., 2014), porcine (Mao et al., 2004) and ovine (Andrade et al., 2005) ovarian tissue.
Furthermore, GCs isolated from human preantral follicles display increased growth in response to EGF in vitro (Roy and Kole, 1998).

These studies suggest that the ErbBs are present in preantral follicles and when activated they support and stimulate preantral follicle development. However, further investigation is required into the presence of ErbB subtypes and dimer combinations formed, the downstream signalling pathways activated and the physiological consequence of EGF signalling on preantral follicle development.

1.6 Androgens

The essential role of androgens in male reproductive development is well characterised. However, it is becoming clear that androgens also play an important role in female reproduction stretching further than as a precursor for oestradiol production.

1.6.1 Androgen Synthesis

The principal circulating androgenic steroids in the female are dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione (A4), testosterone and dihydrotestosterone (DHT), in descending order of serum concentration (Burger, 2002). The pro-androgens DHEAS, DHEA and A4 require conversion to bioactive testosterone or DHT to bind the androgen receptor (AR) and elicit physiological effects. The main sites of androgen biosynthesis in the human are the adrenal glands and the ovaries. The adrenal glands are largely responsible for DHEA and DHEAS production, while A4, testosterone and DHT are synthesised equally from the ovary and adrenals. In the female mouse however, androgens are synthesised predominantly in the ovary. Secreted testosterone circulates in both its free form and bound to proteins such as sex hormone-binding globulin (SHBG) and albumin.

Androgen synthesis in the ovary occurs within the theca cells and favours the Δ\textsuperscript{5} pathway. Initially cholesterol is converted to pregnenolone, catalysed by the enzyme P450 side chain cleavage (P450scc, Cyp11a1). Pregnenolone is converted to DHEA by the enzyme P450c17 (Cyp17), and then to A4 by 3β-hydroxy steroid dehydrogenase (3βHSD). Bioactive testosterone is produced by the conversion of A4 by 17β-hydroxy steroid dehydrogenase (17βHSD). Testosterone can then be reduced by 5α-Reductase 1.
(SRD5A1) or 2 (SRD5A2) to DHT, or aromatised within the GCs to oestradiol by P450arom (Cyp19). The side chain cleavage enzyme and the steroidogenic acute regulatory protein (StAR), which promotes transfer of cholesterol to the inner mitochondrial membrane, are the rate limiting steps for androgen synthesis (Burger, 2002). The classical model of androgen synthesis in the ovary follows the two-cell, two-gonadotropin model (Hillier et al., 1994) (Figure 1.9). The regulation of androgen secretion is under the control of LH signalling on the ovarian theca cells, which stimulates the rate-limiting conversion of cholesterol to pregnenolone. Androstenedione and testosterone then diffuses into the GCs, where they are aromatised to oestradiol or oestrone under the control of FSH signalling.

Figure 1.9 The two-cell, two-gonadotropin model of steroid synthesis in the ovary.

Luteinizing hormone (LH) signalling stimulates the rate-limiting conversion of cholesterol to pregnenolone in the theca cells, catalysed by P450 side chain cleavage enzyme (Cyp11a1), which is then converted to dehydroepiandrosterone (DHEA) by cytochrome P450 17 (Cyp17), then to androstenedione (A4) by 3β-hydroxy steroid dehydrogenase (3βHSD), then converted to bioactive testosterone by 17β-hydroxy steroid dehydrogenase (17βHSD). Testosterone can then be reduced by 5α-Reductase (SRD5A) to dihydrotestosterone (DHT), or aromatised to oestradiol by aromatase cytochrome P450 (Cyp19), regulated by follicle stimulating hormone (FSH) signalling within the GCs.

The concentration of testosterone in follicular fluid has been published in humans. McNatty et al. reported that concentrations ranged between 16.1 ng/ml and 104.4 ng/ml depending on the phase in the menstrual cycle and the size of the follicle (McNatty et al., 1976). Concentration of testosterone was highest in the follicular phase compared to the luteal phase and decreased as the follicle diameter increased. This decrease in testosterone concentration with increasing diameter may be associated with increased utilization of androgens for oestrogen biosynthesis in maturing follicles, as well as a dilution effect caused by accumulating follicular fluid. In the same study
plasma concentrations of testosterone were also measured across the menstrual cycle and recorded between 0.33 ng/ml – 0.51 ng/ml. Interestingly, samples taken from ovarian venous plasma were significantly higher ranging between 0.85 ng/ml and 1.84 ng/ml, indicating testosterone secretion from the ovary. The levels of DHT in human follicular fluid have also been reported but vary substantially between authors. McNatty et al. reported that concentrations of DHT in the follicular fluid were more than 100 ng/ml and far exceeded that of testosterone (McNatty et al., 1979b). However, other authors have reported DHT concentrations were less than 10 ng/ml and much lower than testosterone (Lobo et al., 1985). In various other species including the rhesus monkey (Morgan et al., 1990) and sheep (Tetsuka and Nancarrow, 2007) the concentration of DHT in follicular fluid is often lower than 10 ng/ml. Similarly to testosterone, the follicular fluid concentrations of DHT are reported as approximately 100 times higher than those in plasma.

1.6.2 Androgen receptor signalling

Androgens exert their effects principally through AR, a member of the nuclear receptor superfamily. AR consists of four structurally and functionally distinct domains, the N-terminal transactivation domain (NTD), the deoxyribonucleic acid (DNA)-binding domain (DBD), the ligand-binding domain (LBD), and a short amino acid sequence hinge region that separates the LBD from the DBD and contains part of the NLS (Claessens et al., 2008). AR acts classically as a ligand activated transcription factor, however recently AR has been shown to have a second mode of action, signalling rapidly within the cytoplasm through non classical (non-genomic) mechanisms.

1.6.2.1 Genomic AR signalling

During classical genomic signalling ligand-activated AR directly regulates gene transcription (Gelmann, 2002, Bennett et al., 2010, Mangelsdorf et al., 1995). AR exists in an unbound state in the cytoplasm attached to heat shock proteins (HSP), cytoskeletal proteins and other chaperones (Figure 1.10). Ligand binding induces a conformational change, which results in the dissociation of HSPs to allow binding of proteins such as importin-α, filamin-A, and androgen receptor-associated protein-70 (ARA70), which promote AR stability, nuclear translocation and dimerisation. AR functions most commonly as a homodimer. AR dimers bind to androgen response
elements (ARE) in the promoter regions of target genes to directly regulate transcription. The regulation of gene transcription is dependent on co-regulators, either enhancing (coactivators) or repressing (corepressors) AR activity. Over a hundred AR co-regulators have been identified (Heemers and Tindall, 2007), and their presence and combination in which they are employed is dependent on cell type. Co-regulators have a variety of functions and can act as chaperones that coordinate AR maturation and movement, histone modifiers, coordinators of transcription and DNA structural modifiers. Recruitment of co-regulators including general transcription machinery, results in androgen-regulated gene transcription. This classical pathway of androgens action results in alterations in gene expression and protein production peaking several hours after stimulation. Androgens can dissociate from AR in the nucleus and unbound AR is either shuttled back to the cytoplasm and re-cycled in preparation for further ligand binding, or targeted for proteosomal degradation following ubiquitination by E3 ubiquitin ligase.

1.6.2.2 Non-genomic AR signalling

In recent years it has been reported that the effects of androgens in several target tissues depend upon extranuclear (non-genomic) signalling pathways, reviewed by Foradori et al. (Foradori et al., 2008). Non-genomic signalling was initially discovered through the observation of androgenic effects in cell types that lacked a functional AR. Additionally, some androgenic effects were observed too rapidly to involve changes in gene transcription and were not reduced by the presence of transcription and translation inhibitors. AR non-genomic signalling is characterised by speed, with cellular responses observed within seconds to minutes, indicating a lack of transcription and translation from androgen-responsive genes. These non-classical mechanisms of androgen signalling are thought to be mediated through ARs situated in the cytoplasm near the plasma membrane, and often involve crosstalk with either G-proteins, kinases or growth factor receptors (Revelli et al., 1998, Kousteni et al., 2001, Sen et al., 2011) (Figure 1.10). Androgen exposure has been shown to stimulate rapid release of intracellular calcium and activation of protein kinases such as MAPK, protein kinase A (PKA), AKT and PKC (Foradori et al., 2008). The initiation of these non-genomic signalling pathways may ultimately serve to modulate transcriptional activity of the AR or other transcription factors. The activity of AR and AR co-activators has been shown
to be influenced by direct phosphorylation of MAPK. For example, AR phosphorylation by ERK is associated with enhanced AR transcriptional activity and increased ability to recruit ARA70 (Yeh et al., 1999). Although there are many proposed mechanisms of non-genomic androgen signalling this project focuses on those including interaction with the ErbBs.

Figure 1.10 Overview of AR non-genomic signalling pathways. Androgens can bind and activate the androgen receptor (AR) causing disassociation from heat shock proteins (HSP). AR can translocate to the nucleus to regulate gene transcription. Alternatively, cytoplasmic AR can interact with G protein–coupled receptor (GPCR) proteins, cytoplasmic kinases such as Src, or growth factor receptors including receptor tyrosine kinases (RTK) to elicit rapid response seen in intracellular calcium (Ca\(^{2+}\)) release and activation of second messenger pathways, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT. Non-genomic (dashed grey line) and genomic (solid grey line) signalling may converge within the nucleus to regulate gene transcription. Redrawn and adapted from (Bennett et al., 2010).

1.6.2.3 Non-genomic AR signalling involving ErbBs
Androgen induced non-genomic signalling involving activation of the ErbBs has been shown to promote proliferation and differentiation in the testis. Within the Sertoli cells,
which play a supporting role in germ cell development in the testes similar to GCs in the ovary, testosterone signals through both genomic and non-genomic pathways to support spermatogenesis (Walker, 2010). A review by Walker postulated that many of the target genes regulated by testosterone supporting spermatogenesis did not appear to be regulated directly by AR as they did not contain AREs within their promoter region. The author presented two previously published mechanisms of non-classical testosterone signalling. Firstly, rapid calcium influx shown in isolated Sertoli cells within seconds of androgen stimulation (Gorczynska and Handelsman, 1995). Secondly, isolated Sertoli cells from rat testes display phosphorylation of the cAMP response element binding (CREB) transcription factor and ERK1/2 within just 1 minute of androgen treatment, showing rapid second messenger signalling (Fix et al., 2004). This phosphorylation was not attenuated with inhibitors of transcription or translation, but was decreased with the addition of the AR antagonist flutamide. Another study by the same group by Cheng et al. reported that testosterone activated MAPK in Sertoli cells relied on cytoplasmic tyrosine kinase Src and EGFR activity. The authors attempted to uncover the molecular workings of this non-genomic AR signalling (Cheng et al., 2007a). They found testosterone bound to AR situated close to the plasma membrane, which induced the phosphorylation of the Src within 1 minute. Direct interaction between AR and Src kinase was confirmed by co-immunoprecipitation. Activated Src was shown to promote phosphorylation of EGFR, inducing the activation of the MAPK signalling cascade. Furthermore, addition of a Src kinase inhibitor or an inhibitor which selectively blocks EGFR, AG1478, ablated MAPK signalling. Phosphorylated ERK1/2 activated CREB, which is known to function as a transcriptional co-activator to modify gene expression during spermatogenesis (Fix et al., 2004). Similarly, in LNCaP prostate cancer cells, Migliaccio et al. have proposed a mechanism in which the AR, in conjunction with the oestrogen receptor β (ERβ), binds and activates Src, forming a complex which then activates kinase signalling of the EGFR. The authors showed that AR directly interacted with the SH3 domain of Src within 1 minute (Migliaccio et al., 2000), and inhibition of the SH3 domain reduced AR and Src interaction (Migliaccio et al., 2007).

One alternative mechanism by which steroids may activate the EGFR is through the rapid MMP-mediated shedding of membrane bound precursors of EGFR ligands, such as
HB-EGF, which go on to activate the EGFR extracellularly (Figure 1.11). Similar mechanisms involving steroid stimulated shedding of EGF-like ligands have been reported with oestrogen receptors. E2 stimulation of ERK1/2 phosphorylation in breast cancer cells has been shown to be dependent on the rapid liberation of HB-EGF by Src-induced MMPs (Razandi et al., 2003). Similarly, studies in prostate cancer cells have found that DHT-induced rapid phosphorylation of EGFR and the MAPK cascade could be blocked with the addition of the MMP inhibitor Galardin, which prevented the shedding of EGF-like ligands (Sen et al., 2010). However, studies carried out in Sertoli cells did not support the findings from cancer models. Pre-treatment with either a broad-spectrum MMP inhibitor (GM6001) or an antiserum against HB-EGF, or an antibody (m225) that blocks binding to the EGFR, did not limit androgen-induced ERK or CREB phosphorylation (Cheng et al., 2007a). These studies indicate that rapid activation of EGFR by androgens within Sertoli cells occurs through intracellular mechanism and is not dependent on the shedding of HB-EGF or other external stimuli of the EGFR.

Figure 1.11 Two proposed mechanisms of rapid EGFR stimulation by AR.
Ligand activated androgen receptor (AR) in the cytoplasm may 1) stimulate the rapid shedding of epidermal growth factor (EGF)-like ligands through stimulating matrix metalloproteinase (MMP) activity. The released soluble EGF-like ligands go on to activate the EGFR extracellularly. 2) Alternatively, AR may directly activate cytoplasmic tyrosine kinase Src, which intracellularly activates signalling by the EGFR. Activated EGFR can stimulate signalling through both the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signalling cascades.
1.6.2.4 Membrane AR signalling

The existence of novel membrane androgen binding sites has been suggested by some authors based on detection of androgen binding at the plasma membrane in cells such as osteoblasts (Armen and Gay, 2000) and prostate cancer cells (Kampa et al., 2002). However, the putative membrane receptor has not been purified or cloned. Steroids bound to inert macromolecules such as bovine serum albumin (BSA), which prevent movement across the cell membrane, have been used to investigate the present of membrane binding site. T cells which lack measurable expression of intracellular AR, when treated with testosterone conjugated to BSA rapidly increase intracellular calcium, reviewed by Wunderlich et al. (Wunderlich et al., 2002). This process was not blocked by AR antagonists such as flutamide. These findings may suggest the presence of a novel androgen binding receptor in the membrane.

A subpopulation of the classic AR has been found localised close to the plasma membrane, associated with caveolin-1, a small integral membrane protein (Lu et al., 2001). Additionally, a population of AR has been shown to be palmitoylated (covalent attachment of a fatty acid, primarily palmitic acid), which increases the hydrophobicity of the protein, promoting membrane localization and facilitating caveolin-1 association (Pedram et al., 2007).

1.6.2.5 Physiological importance of non-genomic signalling

There is wide consensus that the classical genomic model of androgen action is responsible for many of the responses to the steroid hormone, while the physiological role of non-genomic signalling is less clear. In LNCaP prostate cancer cells it has been suggested that inhibition of c-Src kinase or MAPK can prevent androgen induced cell cycle progression (Migliaccio et al., 2000), however it is important to note that these effects were observed over a time period of hours and it is therefore difficult to determine the contribution of non-genomic signalling. Nevertheless, it is interesting that many genes regulated by androgens during spermatogenesis do not possess AREs in their promoter region (Walker, 2009). Non-genomic and genomic signalling may cooperate and converge within the nucleus to regulate changes in gene transcription.
1.6.2.6 Non-genomic signalling in the ovary

There has been limited research into the role of non-genomic androgen signalling in developing follicles, however there is emerging evidence that rapid non-genomic signalling may be present in the ovary. Neonatal mouse ovaries display activation of the PI3K/AKT pathway within minutes of exposure to testosterone (Yang et al., 2010). Additionally, non-genomic androgen signalling has also been shown in mammalian oocyte maturation. Studies in mouse oocytes have shown that testosterone can induce maturation independent of transcription involving the activation of MAPK and CDK1 signalling (Gill et al., 2004).

A study from Sen et al., has reported that androgens can promote follicle development independent of transcription through the MAPK pathway, through the up-regulation of the FSHR (Sen et al., 2014). DHT added to a mouse primary GC culture up-regulated FSHR protein but not mRNA. As there was no change in protein degradation with DHT, the authors speculated that DHT promotes FSHR protein expression by enhancing translation through extranuclear AR signalling. Moreover, U0126 reduced DHT stimulation of FSHR implicating the MAPK in this extranuclear signalling. Additionally, the authors found increased phosphorylation of ERK1/2 in mouse GCs following 30 minutes treatment with DHT.

1.6.3 Androgen actions in the ovary

Androgens serve as an essential precursor for oestrogen synthesis in the ovary (Hillier et al., 1994) but also exert androgenic effects, reviewed by Walters (Walters and Handelsman, 2017, Walters et al., 2008). In recent years our understanding of the role that androgens play in the ovary has changed substantially. Traditionally, androgens were considered detrimental to a woman’s reproductive health, implicated in disorders such as polycystic ovarian syndrome (PCOS), however the development of AR knockout mouse models (Shiina et al., 2006, Hu et al., 2004, Walters et al., 2007) have established that androgen signalling can not only improve ovarian function, but is in fact essential to normal follicle development. It is likely that a critical balance exists between low level androgens necessary for normal follicle development and excessive androgen production, which can underlie conditions such as PCOS (Prizant et al., 2014, Pan et al., 2015). The mechanisms behind androgen actions in the ovary and during preantral
follicle development remain unclear, however this area of research has attracted increasing interest in the last few years (Lebbe and Woodruff, 2013, Kimura et al., 2007, Gleicher et al., 2011).

1.6.3.1 Androgen receptor expression in the ovary
AR mRNA and protein expression has been detected at most stages of follicular development. AR has been reported as absent in dormant primordial follicles of rat (Szoltys and Slomczynska, 2000), cow (Hampton et al., 2004), sheep (Juengel et al., 2006), monkey (Hild-Petito et al., 1991) and human (Rice et al., 2007, Suzuki et al., 1994) ovary with little work performed in the mouse. AR expression has been shown to appear in the GCs of activated primary follicles, published in the rat (Szoltys and Slomczynska, 2000), cow (Hampton et al., 2004, Salvetti et al., 2012), pig (Juengel et al., 2006), primate (Hild-Petito et al., 1991) and human (Rice et al., 2007) ovary. Localisation of AR protein in other model species found that AR was most abundant in the GCs of preantral and early antral follicles in primate ovary (Hillier et al., 1997), with strongest expression at the preantral stage in rodent ovaries (Lenie and Smitz, 2009, Tetsuka et al., 1995). Studies undertaken on the primate ovary found most abundant AR expression was located in the GCs of follicles with lesser expression observed in the surrounding thecal and stromal cells (Weil et al., 1998, Hillier et al., 1997). AR expression has been observed to decrease as follicles progress to the pre-ovulatory stage (Hillier et al., 1997), with a gradient of AR staining stronger in the cumulus cells closest to the oocyte and becoming weaker in the mural GCs (Szoltys and Slomczynska, 2000). The distinct spatial and temporal expression of AR gives an indication of the relative importance of androgen signalling throughout follicular development, with particularly high expression seen in the GCs of early stage follicles.

1.6.3.2 AR knock out models
In the 1970s the first models of female androgen insensitivity, found that female mice with homozygous testicular feminization mutation (tfm), which renders AR dysfunctional, resulted in a reduced reproductive lifespan. The ovaries from these mice exhibited a reduction in primordial follicle number and increased follicle atresia (Lyon and Glenister, 1980), providing early evidence that androgen signalling was involved in follicle development. Since then, global and tissue specific AR knockout mice have been
produced using the Cre/loxP system, reviewed by Walters et al. (Walters et al., 2010) (Figure 1.12).

**Figure 1.12 Androgen Receptor knock out mice reproductive phenotypes.**

Global androgen receptor (AR) knock out mice (ARKO), granulosa cell-specific AR knockout mice (GCARKO), oocyte-specific ARKO mice (OoARKO) theca cell-specific AR knockout mice (TCARKO) have been generated and display different reproductive phenotypes. CL, corpus luteum; FSHR, follicle stimulating hormone receptor; IGFR1, insulin-like growth factor 1 receptor; KL, kit ligand; GDF9, growth differentiation factor 9; BMP15, bone morphogenetic protein 15.

Global AR knock out (ARKO) female mice are subfertile and produce fewer pups per litter (Shiina et al., 2006, Hu et al., 2004, Walters et al., 2007). One study also found accelerated follicle depletion with complete loss of follicles by 40 weeks of age (Shiina et al., 2006). Closer examination of the ovaries from ARKO mice revealed disrupted follicular development and increased atresia. Important regulators of follicle growth such as FSH and IGF1R were reduced in ARKO mice (Hu et al., 2004), although follicle diameters at varying stages of development did not appear altered (Walters et al., 2007). Microarray analysis of ovaries also revealed that important factors involved in the oocyte and GC regulatory loop such as KL, BMP15 and GDF9 were reduced (Shiina et al., 2006), which may link to the impaired oocyte health reported in these mice (Hu et al., 2004). Ovulation was compromised in all studies of ARKO mice, with fewer observed
ovulated oocytes and CLs, linked to both impaired antral follicle development but also extra-ovarian defects in the hypothalamic-pituitary regulation.

Two distinct GC-specific AR knockout mice (GCARKO) have been produced, and are both subfertile displaying strikingly similar phenotypes to the female ARKO mice (Sen and Hammes, 2010, Walters et al., 2012). GCARKO mice produce fewer pups per litter and display defective follicle development. Interestingly, numbers of preantral follicles are increased, while number of antral follicles and CLs are decreased, and mice display accelerated follicle depletion (Sen and Hammes, 2010). In both models, GC specific AR deletion resulted in decreased follicular health. Recently, theca cell specific AR knockout mice (TCARKO) have been produced, which show no difference in fertility compared with control littermates (Ma et al., 2017). Also oocyte specific ARKO mice (OoARKO) have been generated and exhibit normal fertility, oestrous cycles and follicle populations (Sen and Hammes, 2010). These ovarian cell specific ARKO mice have helped establish that GCs are a central target for androgen action in the ovary.

1.6.3.3 Animal models of androgen action

Animal models including both in vitro and in vivo studies have provided valuable evidence for the role of androgens during early follicle development. Androgens have been shown to promote primordial follicle activation in both in vitro cultured neonatal mouse ovaries (Yang et al., 2010), and in vivo models which have shown that administration of androgens in both monkeys (Abbott et al., 1998, Vendola et al., 1998) and ewes (Smith et al., 2009) promote initiation of follicular recruitment and stimulation of early follicular growth. Preantral follicles isolated and cultured with testosterone or DHT have shown that androgen exposure significantly enhances preantral follicle diameter and development in the mouse (Murray et al., 1998, Wang et al., 2001, Sen et al., 2014) and in bovine ovarian cortical pieces (Yang and Fortune, 2006), which can be blocked by a non-steroidal AR antagonist, such as bicalutamide (Murray et al., 1998). Additionally, testosterone treatment has been shown to have a stimulatory effect on genes involved in ovarian steroidogenesis, increasing Cyp19 and Cyp11a1 in cultured rat GCs (Wu et al., 2011). However, balance is key as treatment with higher doses of androgens have a negative impact on follicular growth and viability as well as oocyte quality (Romero and Smitz, 2010, Tarumi et al., 2012). Interestingly,
androgens are proposed to have a synergistic relationship with the gonadotropin FSH during preantral follicle development. Testosterone administered to primates increases FSHR mRNA expression in developing follicles (Weil et al., 1999), while DHT increases FSHR mRNA in cultured mouse preantral follicles (Laird et al., 2017). Increased FSHR expression may enhance sensitivity of follicles to FSH, and indeed when both ligands, FSH and DHT, are added in combination they display a synergistic effect on growth (Sen et al., 2014, Laird et al., 2017).

As well as promoting preantral follicle development, a role for androgens in influencing follicle atresia has also been uncovered. Atresia of follicles is elevated in all ARKO and GCARKO mouse models (Hu et al., 2004, Shiina et al., 2006, Walters et al., 2007). Additionally, levels of apoptotic GCs and follicle atresia was decreased following administration of testosterone or DHT to primates (Vendola et al., 1998). Although the mechanisms of androgen on follicle atresia are unknown, testosterone and DHT have recently been found to enhance GC expression of the anti-apoptotic microRNA, microRNA125b, thereby suppressing expression of pro-apoptotic proteins such as BAK1, BMX, BMF and TRP53 (Sen et al., 2014).

1.6.3.4 Androgen interaction with growth factors in the ovary

Androgen has been shown to interact with local ovarian growth factors to regulate preantral follicle development. Recent work produced by our group revealed neonatal mouse ovaries cultured in the presence of DHT displayed a significant increase in IGF1R protein expression, which resulted in elevations in cytoplasmic Foxo3 expression, indicative of increased follicle growth (Laird, unpublished). Furthermore, evidence from in vivo studies conducted in rhesus monkeys has shown that androgen induced follicle growth is attributable to an increase in the expression of the type 1 IGF1R (Vendola et al., 1999). Additionally, stimulation of porcine GCs by IGF1 of GDF9 is enhanced with the addition of DHT (Hickey et al., 2005, Hickey et al., 2004).

1.6.4 Pathologies associated with excessive androgen production: PCOS

PCOS is the most common cause of anovulatory infertility, accounting for over 75% cases in women of reproductive age (Franks, 1995). The syndrome commonly presents as a combination of oligo/anovulation and clinical or biochemical evidence of
hyperandrogenism (Rotterdam, 2004). As well as detrimental effects on fertility, there is also an associated increased risk of metabolic disorders such as Type 2 diabetes (Rotterdam, 2004, Ehrmann, 2005). Currently 5 – 10 % of the female population suffer from PCOS. Despite its high prevalence very little is known of the molecular mechanism underlying the disorder.

1.6.4.1 Mechanism of androgen excess in PCOS

Hyperandrogenism is a hallmark of PCOS, used as one of the Rotterdam criteria for diagnosis (Rotterdam, 2004). Clinical evidence has implicated androgen-mediated actions as central in the development and maintenance of PCOS, as women with high levels of androgens caused by congenital adrenal hyperplasia (Hague et al., 1990) or testosterone treatment in female-to-male transsexuals (Futterweit and Deligdisch, 1986), develop ovarian traits of enlarged, multi-cystic ovaries and theca interstitial hyperplasia indicative of PCOS. Excess ovarian androgen production in women with PCOS has been hypothesised to result from an intrinsic abnormality in the theca cells (Ehrmann et al., 1995, Nelson et al., 2001). Theca cells obtained from the polycystic ovary when cultured display an increase in steroidogenic enzyme activity, particularly Cyp17, and produce on average 20 times more androstenedione than theca cells isolated from control ovaries (Gilling-Smith et al., 1994). Androstenedione is converted to testosterone within the theca cells, resulting in a high androgenic environment surrounding the developing follicles. Antral follicles of polycystic ovaries have been shown to be surrounded by a theca layer of considerably greater cellularity and thickness (Chang, 2007), which may also contribute to androgen excess. Additionally, abnormally increased pituitary LH secretion which is observed with PCOS (Rebar et al., 1976) likely encourages excess ovarian androgen production. Hyperinsulinemia is more common in women with PCOS, and insulin has been shown to augment LH induced androgen biosynthesis (Barbieri et al., 1984), adding to the complex mechanisms underlying androgen excess in PCOS.

1.6.4.2 Dysregulated follicle development in PCOS

Disturbances in ovulation associated with PCOS have been linked to dysregulated follicle development (Franks et al., 2000, Franks et al., 2008). Within the polycystic ovary, the initiation of dormant primordial follicles into a growing state is increased,
together with enhanced growth of the early preantral follicles (Maciel et al., 2004, Franks et al., 2008) (Figure 1.13). Ovarian cortical biopsies taken from patients presenting with polycystic ovaries display a significantly higher proportion of early growing follicles, with a parallel lower proportion of dormant primordial follicles compared with biopsies taken from control women (Webber et al., 2003). However despite the initial increase in growth, follicles arrest at the antral stage of development and fail to ovulate, resulting in the accumulation of many large antral follicles under the ovarian cortex (Franks et al., 2000, Jonard and Dewailly, 2004). The majority of research to date has focussed on the abnormalities during the latter stages of development and ovulation, however, there is growing consensus that abnormalities most likely lay down their roots during preantral follicle development (Franks et al., 2008).

![Normal ovary vs Polycystic ovary](image)

**Figure 1.13 Altered follicle development in the polycystic ovary.**
Ovarian cortical biopsies obtained from women with polycystic ovaries have shown a decrease in the proportion of dormant primordial follicles, with a parallel increase in the number of growing preantral follicles. Follicles in the polycystic ovary become arrested at the antral stage of development and accumulate under the ovarian surface.

Although little is known of the mechanisms underlying the disruption in preantral follicle growth, hyperandrogenism is thought to play a central role (Walters, 2015). Prenatally androgenised female rhesus monkeys and sheep display a reproductive and metabolic phenotype in adulthood similar to that observed in women suffering from PCOS (Forsdike et al., 2007, Abbott et al., 2008, Veiga-Lopez et al., 2011, Abbott and Bacha, 2013). Injections of testosterone or DHT administered to pregnant sheep resulted in greater numbers of early growing follicles within the ovaries of the
developing foetus and the 10 month old females offspring, suggesting follicular recruitment was under androgenic regulation (Smith et al., 2009). Additionally administration of high doses of testosterone to adult rhesus monkeys increased ovarian size and was accompanied by greater numbers of preantral follicles (Vendola et al., 1998). There have also been numerous in vitro studies discussed earlier (see Section 1.6.3.3) demonstrating that cultures of mouse tissue with high testosterone result in increased follicle activation (Yang et al., 2010) and preantral follicle growth (Murray et al., 1998). Intra-ovarian androgen excess is therefore thought to be a major factor in the abnormalities observed in preantral follicle development in PCOS.

1.7 Experimental culture models

Human cortical biopsies containing early follicle stages are extremely rare, therefore in order to investigate the mechanisms controlling primordial follicle activation and preantral follicle development, the mouse was used as a model species. There are some key differences in the mouse and human reproductive systems that should be kept in mind. Firstly, mice are a poly-ovulatory species rather than mono-ovulatory like humans. Secondly, humans have menstrual cycles and shed the endometrial lining each cycle if fertilisation does not occur, in contrast rodents experience a shorter oestrous cycle with no shedding. Thirdly, following years of regular cycling, women experience a reproductive senescence, called the menopause, initiated by the lack of primordial follicles in the ovary, which does not occur naturally in the mouse. However, the short reproductive cycle length observed in mice, has made them a popular animal model for investigation of changes that occur during the reproductive cycle, and mouse ovaries containing small preantral follicles are more easily accessible than human samples. Mice display a similar reliance on the hypothalamus-pituitary-gonadal (HPG) axis for successful reproduction as humans, furthermore many of the intraovarian signalling pathways identified that control early follicle development in the mouse have been replicated in the human. Three mouse culture systems were employed as part of this thesis to examine preantral follicle development. Additionally, our lab is fortunate to have access to samples from control women and women diagnosed with PCOS, in the form of granulosa lutein (GL) cells collected during oocyte retrieval from patients undergoing in vitro fertilisation (IVF) treatment. Although these cells are sourced at a
later phase in follicle development they can provide valuable insights into biological differences in follicular cells from a typically high androgenic ovarian environment.

### 1.7.1 Neonatal ovary culture

Primordial follicles are impossible to isolate from mouse ovary and cannot be cultured, for this reason a whole ovary organ culture system was used as the principal culture method to investigate follicle activation. Whole ovaries dissected from post-partum day 4 (d4) mice, populated largely by the quiescent primordial follicle pool, were cultured on transwell inserts based on a protocol developed by Eppig and O’Brien (Eppig and O’Brien, 1996). The inserts consisted of a porous membrane which allowed medium to coat ovaries in a thin layer. Eppig and O’Brien successfully isolated multilayered follicles from cultured ovaries and showed they were capable of producing mature, fertilizable oocytes. Benefits of this culture system include; maintaining structural support around the developing follicle, allowing communication between follicles and somatic cells as well as preserving inter-follicular signalling between neighbouring follicles. However, the drawbacks include low n numbers per experiment, variability between ovaries and the labour intensive process. Additionally, ovaries could not survive in serum free medium, therefore there are likely some confounding effects from the serum content added to culture medium. Ovary cultures were maintained for up to 6 days, at which stage multiple follicles had initiated growth in the medullary region of the ovary, similar to the wave of follicle activation observed in vivo. Ovaries could be collected and processed for immunohistochemical examination of morphology, activation rate and changes in protein expression.

### 1.7.2 Preantral follicle culture

In order to examine the later stages of preantral follicle development, the culture of whole ovaries was not a viable option, as lack of gas and nutrients within the centre of the larger tissue leads to necrosis. The lab has established a robust culture system for isolated preantral follicles (Fenwick et al., 2013, Hardy et al., 2017, Laird et al., 2017). The follicle culture technique is based on a protocol first developed by Nayudu and Osborn in 1992 (Nayudu and Osborn, 1992). This system involves the culture of individual follicles mechanically isolated from surrounding stromal tissue from post-partum day 16 (d16) mouse ovaries. Follicles are isolated intact and as enclosed units,
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meaning signalling loops and junctional complexes between the GCs and oocytes enclosed in the basal lamina are maintained, but signalling from the surrounding tissue is eliminated, except that from a limited number of theca cells which remain attached to larger follicles. Follicles maintain their spherical three dimensional structure over the culture period. Culturing follicles in individual wells allows for accurate monitoring of growth of each follicle over the culture duration, as follicles can be photographed regularly every 24 hours. Another benefit is that around 40 follicles can be isolated from each ovary, generating high n numbers. However, a limitation of this culture technique is the extremely limited amount of material available for analytical studies following culture. The immature preantral follicles are not yet dependent on gonadotropin signalling and remain healthy and intact without FSH for over 72 hours in culture. Additionally, follicles survive without serum, preventing confounding effects of adding serum to the medium. Murray and Spears demonstrated that cultured preantral follicles were capable of forming an antrum and successfully ovulating in vitro with hormonal stimulation (Murray and Spears, 2000). Following culture, healthy follicles can be collected and taken for a range of analytical studies to uncover changes in gene and protein expression and alterations in proliferation and apoptosis.

1.7.3 GC culture

To investigate rapid changes in second messenger signalling pathways a GC monolayer culture was established. GCs were extracted from post-partum day 26 (d26) mouse ovaries that consisted of primarily preantral and small antral follicles. Ovaries were punctured with fine needles and then pressed to release GCs. This method of isolation was first established in rats (Lederer et al., 1995) and later used successfully in mouse ovaries (Peluso and Pappalardo, 2004). The large number of cells needed for sufficient protein lysate for western blotting, meant establishment of cultures was highly labour intensive and required two people working in parallel isolating cells for each experiment. For this reason, n numbers were relatively low. The main benefit of this culture system was that cells could be easily exposed to androgen stimulation for short periods and there was sufficient protein collected so that activation of downstream phosphorylation events could be investigated through western blotting. However, there were also drawbacks as removal of GCs from the follicular structure induced
spontaneous luteinisation. A detailed overview of the optimisation of the GC culture procedure and methods to prevent spontaneous luteinisation is included in Chapter 8.

1.7.4 Human GL cell culture

Human GL cells were isolated and cultured as previously described by Bonser et al., (Bonser et al., 2000). Samples were divided into normal morphology ovaries with regular cycles and women with polycystic ovaries with irregular cycles. An advantage of using this model is that it makes use of cells that are typically a waste product of the IVF procedure and would otherwise be discarded. Another benefit of using these cells is the abundance of material available compared to mouse GC cultures which can be utilised for molecular analysis as well as protein quantification.

1.8 Summary and Hypothesis

The signalling pathways that regulate primordial follicle activation and preantral follicle development are complex and often interconnected. The hypothesis of this project was that the EGF family plays a role in regulating the preantral stages of follicle development, and androgens modulate follicle development through interaction with the EGF family.

1.9 Aims

- Identify which EGF-like ligands and ErbB receptors are present in the ovary and quantify their relative expression during follicle development.
- Investigate the role of the EGF family during preantral follicle growth.
- Investigate the role of the EGF family during primordial follicle activation.
- Examine the effects of androgen signalling on preantral follicle development.
- Explore crosstalk between the androgen and EGF signalling pathways in the preantral follicle.
- Investigate evidence for non-genomic androgen signalling in preantral follicles.
Chapter 2 Materials and methods

2.1 Tissue collection

2.1.1 Mouse tissue collection

All mice were housed in accordance with the Animals (Scientific Procedures) Act of 1986 and associated Codes of Practice. Female mice (C57BL/6) were killed by cervical dislocation at 4, 16, 26, 28 and 42 days post partum (Envigo, Huntingdon, UK). Whole ovaries and positive control tissue (liver, lung, large intestine, skin, eye and oviduct) were collected in plain Leibovitz's L-15 medium (Cat. No 11415-049; Gibco). Connecting fat, oviduct and ovarian bursa were removed from ovaries under a dissection microscope using insulin needles (Cat. No U-100; Terumo) in L-15 medium. Whole ovaries and organs were either snap frozen in liquid nitrogen for RNA extraction, fixed in 10% neutral buffered formalin solution (Cat. No HT5012; Sigma-Aldrich) for immunohistochemistry, washed in phosphate-buffered solution (PBS; 137 mM NaCl, 3 mM KCl, 2 mM KH2PO4, 10 mM Na2HPO4; pH 7.4) and snap frozen in radioimmunoprecipitation assay buffer (RIPA; Cat. No 89900; Thermo Scientific) with a cocktail of protease and phosphatase inhibitors (1:100; Cat. No 78441; Thermo Scientific) for protein extraction, or moved to minimum essential medium alpha (MEM-α; Cat. No 22561-021; Gibco) for tissue culture.

2.1.2 Mouse ovary dissection

2.1.2.1 Follicle isolation

Preantral follicles (75 - 130 μm diameter, 2-3 layers of GCs) were mechanically isolated from d16 mouse ovaries in L-15 medium supplemented with 1% (w/v) bovine serum albumin (BSA; Cat. No A7030; Sigma-Aldrich) using disposable acupuncture needles (30mm x 0.25; AcuMedic). Isolated follicles with an intact basement membrane and centrally located oocyte (Figure 2.1A) were either taken for further culture or snap frozen in liquid nitrogen in MEM-α for subsequent RNA extraction or RIPA buffer with protease and phosphatase inhibitors for protein extraction.
2.1.2.2 Oocyte isolation
Oocytes (40 - 70 µm diameter) were collected from d16 mouse ovaries during the follicle isolation process in L-15 medium supplemented with 1% (w/v) BSA (Figure 2.1B). Oocytes were cleaned of attached GCs by repeated pipetting. Oocytes were snap frozen in liquid nitrogen in either MEM-α or RIPA buffer with protease and phosphatase inhibitors.

2.1.2.3 GC isolation
GCs were isolated from d26 ovaries in L-15 medium supplemented with 0.05% (w/v) BSA. GC isolation was achieved by puncturing antral follicles with acupuncture needles and gently pressing the ovary to encourage the extrusion of GCs (Figure 2.1C). Aggregates of healthy GCs were collected and taken for culture or snap frozen in MEM-α for RNA extraction.

![Figure 2.1](image)

**Figure 2.1 Isolated mouse follicles, oocytes and GCs.**
Representative images of mouse A) follicles, B) oocytes and C) GCs taken directly after isolation procedure. Scale bar = 100 µm.

2.1.3 Human GL collection
Samples were collected from women undergoing routine IVF at Hammersmith IVF clinic under a licence from the United Kingdom Human Fertilization and Embryology Authority. Written informed consent was obtained as well as ethical committee approval granted by Hammersmith and Queens Charlotte’s Research Ethics Committee (reference number 08/H0707/152 and 10/H0707/2). Anonymised clinical data concerning diagnosis of PCOS, ovarian morphology and IVF stimulation protocols was provided by the clinic. Women received either hCG (Ovitrelle or Pregnyl), GnRH agonist
Buserelin) or kisspeptin-54 as a maturation trigger. Samples were divided into women with normal morphology ovaries, defined by ultrasound, with regular cycles (Control) and women with polycystic ovaries with a history of irregular menstruation or amenorrhoea (PCOS).

Follicular aspirate was obtained via transvaginal, ultrasound-guided oocyte retrieval. Aspirates were transported to the research laboratory and GL cells were extracted from the follicular aspirate. Aspirates were initially centrifuged at 1,000 rpm for 5 minutes to pellet all suspended cells. Aspirate was removed and pellets were reconstituted in medium 199 (M199; Cat. No 22340-020; Gibco) and layered onto a Percoll gradient (Cat. No 17-0891; GE Healthcare). Mixture was centrifuged at 16,000 rpm for 30 minutes to separate red blood cells from GL cells. Suspended GL cells at the Percoll interface were collected and washed in Dulbecco’s PBS (DPBS; Cat. No 14190-144; Gibco), before a final centrifugation 1,000 rpm for 5 minutes. Pelleted cells were re-suspended in 200 µl of M199 medium and either frozen at -80°C for later RNA extraction or taken for cell culture.

2.2 Tissue culture

2.2.1 Mouse follicle culture

Isolated preantral follicles were transferred singly into individual wells in a 96-well plate (Cat. No 167008; Thermo Scientific) containing 100 µl MEM-α medium supplemented with 0.1% (w/v) BSA, 75 µg/mL penicillin (Cat. No PENK; Sigma-Aldrich), 100 µg/mL streptomycin sulphate (Cat. No S6501; Sigma-Aldrich), 5 µg/mL insulin, 5 µg/mL transferrin and 5 ng/mL sodium selenite (ITS; Cat. No I1884; Sigma-Aldrich). Follicles were cultured for 72 hours in a humidified incubator in 5% CO₂ at 37°C. Follicles were cultured in a range of experimental treatments (see Section 2.2.5). At 24 or 72 hours healthy follicles were fixed in 10% neutral buffered formalin, pooled in groups of 10-15 follicles and moved into 2% (w/v) low melting point agarose (Cat. No A9045; Sigma-Aldrich), alternatively follicles were snap frozen in RIPA buffer with protease and phosphatase inhibitors for protein analysis or in MEM-α medium for molecular studies.
2.2.2 Mouse ovary culture

Whole ovaries were dissected from d4 mice and isolated from connecting ovarian bursa and oviduct. Ovaries were cultured on Costar Transwell 3 μm pore polycarbonate membrane inserts in 6 well plates (Cat. No 3414; Corning). Each well contained 1.5 mL of Waymouth’s medium (Cat. No 31220-023; Gibco) supplemented with 0.3% (w/v) BSA, 0.23 mM sodium pyruvate (Cat. No 11360-070; Gibco), 75 μg/mL penicillin, 10 μg/mL streptomycin sulphate, and 2% (v/v) fetal bovine serum (FBS; Cat. No 12359802; GE Healthcare) so that each ovary was covered by a thin meniscus of medium. Every 72 hours, half the culture medium (750 μl) was refreshed. Cultures were maintained for 6 days in a humidified incubator in 5% CO₂ at 37°C (Figure 2.2). Culture medium was supplemented with the range of experimental treatment (see Section 2.2.5). At the completion of culture, ovaries were fixed in 10% neutral buffered formalin solution, moved into 2% (w/v) low melting point agarose and processed for histology.

![Figure 2.2 Cultured d4 mouse ovaries.](image)

Representative images of d4 mouse ovaries over culture, photographs taken at day 0, 2, 4 and 6. Scale bar = 300 μm.

2.2.3 Mouse GC culture

GCs were isolated as previously described (see Section 2.1.2.3) from d26 murine ovaries. Isolated cells were transferred to a 50 mL Falcon tube (Cat. No 525-0402; VWR) containing culture medium consisting of MEM-α medium supplemented with 0.1% (w/v) BSA, 75 μg/mL penicillin, 100 μg/mL streptomycin sulphate, 2% (v/v) FBS. The Falcon tube was left in a humidified incubator between ovary processing. GCs were centrifuged for 5 minutes at 1,000 rpm then resuspending in 1 mL culture medium. Cells were counted with a haemocytometer under a light microscope. Cell viability was assessed using the dye exclusion test and Trypan blue solution (Cat. No 15250-061; Gibco), viable cells did not take up the impermeable dye, but dead cells are permeable.
and stained blue. Cell counts were repeated in triplicate and cells plated at 0.5 - 1 x 10^5 per well in a 12 or 24 well plate (Cat. No 3512 and 3527 respectively; Corning). Cultures were maintained for up to 1 week in a humidified incubator in 5% CO_2 at 37°C. Optimisation of the GC culture conditions are described in detail in Section 8.2.1. Attachment factor (Cat No. S-006-100; Gibco) was used as part of the optimisation. Cells were treated with various experimental treatments (see Section 2.2.5). Following treatment cells were collected for protein analysis and either 1) washed in ice cold PBS and lysed in RIPA buffer with protease and phosphatase inhibitors, or 2) fixed in 4% paraformaldehyde (Cat. No 28906; Thermo Scientific). For molecular analysis, cells were washed in PBS, scraped and snap frozen and stored at -80°C until further processing.

2.2.4 Human GL cell culture

Cells were collected and isolated as previously described in Section 2.1.3. To remove any red blood cell contamination, 2mL of diluted RBC lysis buffer (1:10; Cat. No 420301; BioLegend) was added to isolated GL cells for 5 minutes at room temperature. Cells were spun down at 1,000 rpm for 5 minutes, lysis buffer aspirated and cells reconstituted in 1mL of culture medium consisting of Dulbecco’s modified eagle medium (DMEM; Cat. No 11885-084; Gibco) supplemented with 0.1% (w/v) BSA, 75 µg/mL penicillin, 100 µg/mL streptomycin sulphate, and 10% (v/v) Charcoal/Dextran treated FBS (Cat. No SH30068; GE Healthcare). Cell counts were performed in triplicate using a haemocytometer and viability assessed with Trypan blue staining under a light microscope. Cells were plated at 2 - 3 x 10^5 in 24 well plates. Cells were left to attach overnight, then washed in warm PBS and medium replaced with glucose, glutamine and phenol free DMEM medium (Cat. No A1443001; Gibco) supplemented with 0.1% (w/v) BSA, 75 µg/mL penicillin, 100 µg/mL streptomycin sulphate, and 0.5% (v/v) Charcoal/Dextran treated FBS at 24 or 48 hours depending on degree of cell confluence. At the completion of culture, cells were washed in ice-cold PBS and RIPA buffer with a cocktail of protease and phosphatase inhibitors was added to each well.
2.2.5 Experimental treatments

Culture medium was supplemented with a range of ligands and various receptor and downstream signalling inhibitors. The preparation of these treatments and their modes of actions are shown below.

2.2.5.1 EGF

Receptor grade EGF was supplied as a powder (Cat. No 01-102; Millipore) extracted from mouse submaxillary glands. EGF powder was reconstituted in MEM-α medium alone (no supplements) at 10 µg/ml, and stored at -20°C in 10 µl aliquots. A dose response (5, 10 and 100 µg/ml) was performed previously by PhD student Victoria Atess (Atess, 2015), finding maximal effect on follicle growth at 10 ng/ml, therefore this dose was used for all further experimentation.

2.2.5.2 DHT

5α-Androstan-17β-ol-3-one (DHT) was supplied as a powder (Cat. No A8380; Sigma-Aldrich) and reconstituted at 10 mM in molecular grade ethanol (Cat. No 437433T; VWR) and stored at -20°C in 10 µl aliquots. Working solutions were prepared by serial 1:1000 dilutions to 10 nM in culture medium. A dose response was performed previously (1, 10 and 100 nM) which found maximal effect on follicle growth at 10 nM (Laird et al., 2017), therefore all further experimentation used this dose of DHT with ethanol added to all appropriate vehicle controls. The concentration 10 nM DHT was chosen for all further studies, as this concentration is similar to the approximate physiological tissue concentrations of DHT in follicles. As described in Section 1.6.1, given the variability of DHT concentrations reported in follicular fluid, 10nM was chosen to correspond to reports of 2.4 – 5 ng/ml (8.2 - 17.2 nM) in ovine follicles (Tetsuka and Nancarrow, 2007) and < 10 ng/ml (34.4 nM) in human follicles (Lobo et al., 1985).

2.2.5.3 AG1478

The specific EGFR inhibitor AG1478 was supplied as a powder (Cat. No 658548; Calbiochem) and reconstituted to 10mM in dimethyl sulfoxide (DMSO; Cat. No D12345; Invitrogen) and stored at -20°C in 10 µl aliquots. Previously a dose response was performed in the absence of EGF (2, 5, 10 µM) and the presence of EGF (5 and 10 µM) by
Victoria Atess (PhD thesis, 2015), which found maximal inhibitory effect of EGF actions at 10 µM. Therefore 10 µM was used for all further experimentation and DMSO was added to all appropriate vehicle controls. AG1478 is a cell permeable selective and reversible inhibitor of EGFR kinase activity. The inhibitor is ATP-competitive that binds to EGFR ATP binding sites with an IC$_{50}$ value of 3 nM in cell-free assays.

### 2.2.5.4 ErbB2 inhibitor ii

ErbB2 inhibitor ii (Cat. No 324732; Calbiochem) was supplied as a powder and reconstituted to 5 mg/ml (19.06mM) in DMSO and stored at -20°C in 10 µl aliquots. A dose response (0.5, 1, 5, 10 µM) was performed by Victoria Atess (PhD thesis, 2015) and found no change in follicle growth. As the reported IC$_{50}$ value for ErbB2 inhibitor ii is 6.6 µM for phosphorylation, but 30.9 µM for cell growth (Cheng et al., 2007b), a higher dose response was performed (20 µM and 40 µM), as described in Chapter 4. ErbB2 inhibitor ii is a cell-permeable triazole compound that targets the ATP-binding site of ErbB2/HER2 and reduces the phosphorylation of ErbB2.

### 2.2.5.5 U0126

U0126 (Cat. No 662005; Calbiochem) and its inactive analogue U0124 (Cat. No 662006; Calbiochem) were both supplied as powders and reconstituted to 10mM stocks in DMSO and stored at -20°C in 10 µl aliquots. A dose response for U0126 (0.1, 1 or 10 µM) was performed, as described in Chapter 4. U0126 functionally antagonizes AP-1 transcriptional activity via non-competitive inhibition of the dual specificity kinase MEK1 and MEK2 with IC$_{50}$ of 72 nM and 58 nM respectively in cell free assays, and therefore blocks signalling through the MAPK cascade.

### 2.2.5.6 LY294002

The PI3K inhibitor LY294002 (Cat. No L9908; Sigma-Aldrich) was supplied in powder form and reconstituted in DMSO to 10mM stock and stored at -20°C in 10 µl aliquots. A dose response was performed with LY294002 (5 and 10 µM), as described in Chapter 7. LY294002 is a cell permeable inhibitor which inactivates AKT/PKB with an IC$_{50}$ value of 1.4 µM blocking PI3K-dependent AKT phosphorylation (Vlahos et al., 1994), and therefore prevents signalling through the PI3K cascade.
2.2.5.7 Flutamide

Flutamide (Cat. No F9397; Sigma-Aldrich) was supplied as a powder and reconstituted in 100% molecular grade ethanol to make a 20 mM stock solution and stored at -20°C in 10 µl aliquots. A dose response with flutamide (10, 15 and 20 µM) in combination with DHT (10nM) found that 20 µM was sufficient to significantly inhibit DHT stimulated follicle growth in vitro (Laird et al., 2017), therefore this concentration was used in all further experimentation. The anti-androgen flutamide acts as a selective antagonist of the AR, competing with androgens (such as testosterone and DHT) for AR binding.

2.2.5.8 PP2

The Src inhibitor PP2 (Cat. No; 529573; Calbiochem) was supplied as a powder and reconstituted to 10mM stock solution in DMSO and stored at -20°C in 10 µl aliquots. Dose responses performed in mouse ovarian tissue found 10 μM PP2 was maximally effective in theca-interstitial cell cultures (Chaturvedi et al., 2008), therefore the concentration of 10 μM PP2 was chosen for all further studies. PP2 acts as a potent, reversible, ATP-competitive, inhibitor of the Src family kinases, inhibiting Lck/Fyn/Hck/Src with an IC_{50} of 4 nM, 5 nM, 5nM and 100nM respectively in cell-free assays.

2.3 Sample preparation

2.3.1 RNA extraction and cDNA synthesis

2.3.1.1 RNA extraction of whole mouse tissues and human GL cells

Total RNA was extracted from ovaries and positive control tissue (liver, lung, large intestine, skin and eye) as well as human GL cells using a RNeasy mini kit (Cat. No 74104; Qiagen) in accordance with the manufacturer’s instructions. The kit included buffers RLT, RW1 and RPE and microcolumns subsequently described. In brief, tissue was first homogenised, either with a pestle and mortar or homogeniser, in an appropriate volume of RLT buffer (200 µl per whole ovary) with 1% β-mercaptoethanol (Cat. No M3148; Sigma-Aldrich). An equal volume of molecular grade 70% ethanol was added to the lysate and the combination was transferred to an RNeasy column. Columns were centrifuged for 15 seconds at 10,000 rpm and flow-through discarded. The collected RNA was washed with 350 µL RW1 buffer and spun for 15
seconds at 10,000 rpm. Incubation with DNase digestion mix (Cat. No 79254; Qiagen) for 15 minutes removed contaminating DNA. DNase solution was prepared as a mixture of 10 µl DNase I and 70 µl of RDD buffer, included in DNase kit, for each sample. A second wash with 350µl buffer RW1 was performed and columns spun for 15 seconds at 10,000 rpm. Columns were washed with 500 µl RPE buffer and spun for 15 seconds, before a final wash with 500 µl RPE buffer and spun for 2 minutes both at 10,000 rpm. To dry column membranes and remove ethanol contamination, columns were spun with lids open at full speed for 1 minute. RNA was eluted in 20 µl of RNase-free water (Cat. No AM9937; Ambion, Invitrogen) added directly to membrane and centrifuged at 10,000 rpm for a final 1 minute. RNA was either processed immediately for complementary DNA (cDNA) synthesis or stored at -80°C.

2.3.1.2 RNA extraction of mouse isolated follicle, oocytes and GCs

Total RNA was extracted from isolated follicles, oocytes and GCs using a RNeasy Micro Kit (Cat. No 74004; Qiagen) as per manufacturers instructions. The kit included buffers RLT, RW1 and RPE, carrier RNA and microcolumns subsequently described. Samples were lysed in 75 µl RLT buffer with 1% β-mercaptoethanol and vortexed to thoroughly homogenise sample. Carrier RNA (5 µl) was added to lysate and the combination was vortexed for a further minute. Lysate was combined with an equal volume of 70% ethanol and transferred to a microcolumn. In order to bind RNA to the membrane, columns were centrifuged for 15 seconds at 10,000 rpm and flow-through discarded. RNA was washed with 350 µl RW1 buffer and spun for 15 seconds at 10,000 rpm before DNase digestion as previously described (Section 2.3.1.1). Further washes were performed with 350 µl RW1 buffer and 500 µl RPE buffer and columns spun each time for 15 seconds at 10,000 rpm. A final wash with 80% ethanol was followed by 2 minutes centrifugation at 10,000 rpm. To dry membranes columns were spun with lids open at full speed for 5 minutes. RNA was eluted in 14 µl of RNase-free water added directly to membrane and centrifuged at 10,000 rpm for 1 minute. Again, RNA was either processed immediately for cDNA synthesis or stored at -80°C.

2.3.1.3 RNA quantification and quality analysis

The concentration and integrity of RNA extracted from whole tissue and human GL cells was analysed using a Nanodrop spectrophotometer (Nanodrop ND-1000, Thermo
Scientific). Due to the insufficient quantity of RNA extracted from murine isolated follicles, oocytes and GCs, RNA was instead analysed by Agilent 2200 TapeStation. RNA samples were combined with high sensitivity RNA screentape sample buffer (Cat. No 5067-5580; Agilent Technologies) and loaded along with a high sensitivity RNA ScreenTape (Cat. No 5067-5579; Agilent Technologies). Sample analysis was completed using 2200 TapeStation Controller Software (Agilent Technologies). Only samples that scored a RIN score above 8 were used for further experimentation.

2.3.1.4 cDNA synthesis
RNA was concentrated and converted to cDNA using a SuperScript IV reverse transcriptase kit (Cat. No 18091050; Invitrogen), which included all following reagents, in accordance with the manufacturer’s guidelines. In brief, up to 500 ng of RNA extracted from whole tissue, isolated follicles, oocytes and GCs was combined with 10mM dNTP mix and 50 µM random hexamers and made up to 13 µl with nuclease free H2O. The reaction mix was heated to 65 °C for 5 minutes then cooled on ice for at least 1 minute. Combined 4 µl of 5x SSIV buffer, 1 µl of 100 nM DTT, 1 µl of RNaseOUT recombinant RNase inhibitor and 1 µl of SuperScript IV Reverse Transcriptase (200 U / µl) was added to reaction to make a final volume of 20 µl. The reverse transcription mixture was incubated at 23 °C for 10 minutes, 50 °C for 10 minutes and finally 80°C for 10 minutes. The resultant cDNA was diluted to desired concentration in nuclease free H2O and stored at -20 °C.

2.3.2 Protein extraction and quantification
2.3.2.1 Protein extraction from whole mouse tissue
Whole ovaries and positive control tissue (mouse lung and liver), which had been snap frozen in RIPA buffer supplemented with cocktail of protease and phosphatase inhibitors, were thawed and homogenised. Positive control tissue was broken apart with an electric homogeniser, while ovaries were disrupted with a pestle and mortar and sterile scissors. Protein lysate was spun for 15 minutes at 13,000rpm to remove cell debris and the supernatant was collected and stored at -80°C until further use. The pellet was discarded.
2.3.2.2 Protein extraction from mouse follicles, oocytes and untreated GCs

In order to investigate rapid phosphorylation events in isolated preantral follicles, follicles were isolated and pooled into groups of 20 in 50 µl in MEM-α in low protein binding Eppendorf tubes (Cat. No 022431064; Eppendorf). Follicles were treated with ligand or inhibitor of interest before 100 µl of ice-cold PBS was added. Follicles were centrifuged at 1,000 rpm for 2 minutes and PBS gently removed. This wash step was repeated twice and finally 50 µl RIPA buffer with protease and phosphatase inhibitors was added and follicles were snap frozen in liquid nitrogen. Protein from treated follicles, as well as untreated follicles, oocytes and mouse GCs was extracted by vortexing for 2 minutes, followed by centrifuged at 13,000rpm for 2 minutes and then sonication in a 4°C water bath for 3 cycles of 30 seconds.

2.3.2.3 Protein extraction from mouse and human cultured cells

Cell culture dishes were placed on ice and washed 3 times in chilled PBS. PBS was aspirated and ice-cold RIPA buffer with protease and phosphatase inhibitors was added in appropriate volume to each well. Cells were disrupted with a cell scraper (Cat. No CC7600-0220; CytoOne) and cell suspension was moved to low protein binding Eppendorf tubes. The cells were left to lyse for 15 minutes on ice before centrifugation at 4°C for 15 minutes at 13,000rpm to remove cell debris. Supernatant was collected and stored at -80°C until further use and the pellet was discarded.

2.3.2.4 Protein quantification

Total protein concentration was determined with a Pierce BCA Protein Assay Kit (Cat. No 23225; Thermo Scientific). Standards were prepared by diluting BSA (0 - 2000 µg/ml) in RIPA buffer and plated alongside samples on a 96 well plate. Standards and samples were plated in duplicate, then 200 µl of working reagent was added to each well and plate was mixed and incubated at 37°C for 30 minutes. The intensity of colour change was quantified by measuring absorbance at 562 nm on an OPTImax microplate reader (Molecular Devices) using Softmax Pro v4.8 software and concentration of samples was calculated through comparison with the known BSA standards.
2.3.3 Tissue fixation, processing and embedding

2.3.3.1 Tissue fixation
Cultured ovaries and isolated follicles were fixed in 10% neutral buffered formalin solution for 3 hours and 30 minutes respectively. Fixed tissue was washed in PBS then moved into 2% (w/v) low melting point agarose drops on a coverslip on a heated stage. Coverslips were then allowed to cool and agarose to set. Follicles were stained with nuclear fast red solution (Cat. No N3020; Sigma-Aldrich) while in agarose drops making them clearly visible for subsequent sectioning. Agarose drops were moved into 70% ethanol (Cat. No 20821.330; VWR) and stored until further use. Whole untreated ovaries were fixed overnight in 10% neutral buffered formalin, washed in PBS and moved to 70% ethanol. Mouse GCs cultured on coverslips were fixed in 4% paraformaldehyde or 10% neutral buffered formalin solution for 10 minutes at room temperature, then washed and stored in PBS before staining.

2.3.3.2 Tissue processing, embedding and sectioning
Untreated whole ovaries as well as cultured ovaries and follicles in agarose drops were processed through increasing concentrations of ethanol: 70% for 1 hour, 90% for 1 hour, 100% ethanol for 3 x 1 hour, then placed in Histoclear (Cat. No HS-200; National Diagnostics) overnight. The following day samples were incubated in Histoclear again for 1 hour, then embedded in paraffin wax (Cat. No 36107; VWR) in clear disposable base moulds (Cat. No 03015; Surgipath) for 2 hours at 65 °C. Plastic embedding cassettes (Cat. No M480-2; Simport) were added and paraffin blocks were allowed to cool over ice. Tissue was serially sectioned (5 µm) using a Leica RM 2135 microtome, mounted on Superfrost glass slides (Cat. No 631-0108; VWR) and dried overnight at 37°C.

2.4 Sample analysis
2.4.1 Polymerase chain reaction (PCR)
Presence or absence PCR screens were performed on cDNA extracted from ovarian tissue samples. Primers were designed (Table 2.1) or purchased predesigned (Sigma-Aldrich; KiCqStart Primers) toward desired transcripts. KAPA 2G Fast Readymix (Cat. No KK5102; Kapa Biosystems) was combined with 500 nM primer mix, template cDNA and H₂O as shown in Table 2.2. cDNA was amplified in TC-3000 thermal cycler (Techne),
subjected to an initial 95 °C denaturation step for 2 minutes, then 35 cycles of denaturation (95°C for 15 seconds), primer annealing (58 - 62°C for 15 seconds) and extension (72°C for 1 second) then a final extension step of 72°C for 1 minute. Genes specific to either oocytes (Gdf9) or GCs (Amh) were included as positive controls to confirm samples were not contaminated with unintentional cells. PCR products were separated according to size on a 2% agarose gel (Cat. no 9539; Sigma-Aldrich) supplemented with 0.2 µg/ml ethidium bromide (Cat. No E1385; Sigma-Aldrich) made up in 100 ml TAE buffer (40 mM Tris Base, 20mM glacial acetic acid, 1mM EDTA (pH 8.0)) by gel electrophoresis at 100V for 1 hour. Ethidium bromide bound DNA was visualised under Ultra Violet (UV) light in an Alpha Imager mini (Alpha Innotech).

**Table 2.1 Primer sequences**

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<th>Product size (bp)</th>
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## Chapter 2: Materials and methods

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Table 2.2 PCR mastermix per reaction

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<td>Reverse primer (10 µM)</td>
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<td>Nuclease free H₂O</td>
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<td><strong>Total Volume</strong></td>
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2.4.2 Quantitative RT-PCR

Quantitative PCR was performed using SYBR green detection to measure transcript concentrations. In order to quantify gene expression relative to experimental controls the ΔΔCt method was used. Absolute abundance of EGF-like ligands and ErbB receptor transcripts in mouse tissue was assessed through the standard curve method.

2.4.2.1 ΔΔCt method

Quantitative PCR was performed by combining KAPA SYBR FAST mastermix and ROX (Cat. No KK4602; Kapa Biosystems), primer mix and template cDNA (Table 2.3). To each well of a 384 well plate (Cat. No 4309849; Applied Biosystems) 19 µl of mastermix and either 1 µl of template cDNA or 1 µl of H₂O control were added. Adhesive PCR plate seals (Cat. No 4311971; Applied Biosystems) were applied and the plate was briefly spun down at 1400 rpm for 2 minutes. Housekeepers and samples were both run in duplicate. Quantitative PCR cycling was performed on an Applied Biosystems 7900HT Fast instrument. Reaction mix was subjected to an initial 95°C activation step for 3 minutes, then 40 cycles: denaturation (95°C for 3 seconds), primer annealing (58 - 62°C for 20 seconds), extension (72°C for 1 second) and an amplicon-specific fluorescence acquisition reading (74 - 76°C for 10 seconds). Melt curve analysis was performed on every sample to ensure product-specific amplification. ATP5B (Primer Design) and GAPDH (Primer Design) were both used as internal reference genes and the geomean determined. Expression levels were normalised to the geomean of the internal reference genes and calculated as fold change relative to experimental control using the 2-ΔΔCT method (Livak and Schmittgen, 2001). Data was either presented as ΔΔCt values
displayed as fold change from experimental control or data was log$_2$ transformed. RT-PCR products were separated by electrophoresis as described previously in Section 2.4.1 and ethidium bromide bound DNA was visualised under UV light to ensure correct amplicon size in base pairs (bp; Table 2.1).

### Table 2.3 Quantitative RT-PCR mastermix components per reaction

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</table>

#### 2.4.2.2 Standard curve method

For absolute transcript quantification, standards were prepared from RT-PCR products amplified from positive control tissue known to express high levels of the desired gene (Lung: *Egfr, Erbb2, Erbb3, Erbb4*; Eye: *Epgn, Ereg, Areg*; Adult mouse ovary: *Egf, Hbegf, Tgfa*). Purification of PCR products was performed with PCR purification columns (Cat. No 28004; Qiagen). Products were run on 2% agarose gels containing 0.2 µg/ml ethidium bromide and extracted using NucleoSpin Gel and PCR Clean-up columns (Cat. No 740609.50; Macherey-Nagel). All ErbB receptor and EGF-like ligand PCR products were sequenced and confirmed by DNA sequence analysis (Genewiz, UK). Nanodrop spectrophotometer was used to determine the concentration of each purified sample and standards of known concentrations were prepared up to 10 orders of magnitude (1:75 serial dilutions) with nuclease free H$_2$O. Quantitative PCR was performed with cDNA from isolated follicles combined with KAPA SYBR FAST, ROX High and primer mix (Table 2.3). Each standard was run in triplicate and samples in duplicate. Amplification was carried out using StepOnePlus Real-Time PCR System (Applied Biosystems) with cycling as described above in Section 2.4.2.1. Melt curve analysis was performed on every sample to ensure product-specific amplification. Ct values were compared against known standard curve values to derive absolute mRNA quantity (femtograms per follicle).
Chapter 2: Materials and methods

2.4.3 Western blotting

2.4.3.1 Gel electrophoresis

Protein lysate samples of equal concentration were mixed with Bolt LDS Sample Buffer (4x; Cat. No B0007; Invitrogen) and Bolt reducing agent (10x; Cat. No B0009; Invitrogen), then made up to 40 µl with deionised water. Samples were denatured by heating to 70°C for 10 minutes. Reduced samples were loaded into precast NuPAGE® Novex® Bolt 4 - 12% Bis-Tris (1.0 mm x 10 well) gels (Cat. No NW04120BOX; Invitrogen) along with SeeBlue Plus2 pre-stained protein ladder (Cat. No LC5925; Invitrogen). Protein was resolved by gel electrophoresis at 200V for 30 minutes with 1X Bolt MES SDS running buffer (Cat. No B0002; Invitrogen) in a Mini Gel Tank (Cat. No A25977; Invitrogen).

2.4.3.2 Western blotting

Resolved proteins were transferred onto an ECL nitrocellulose membrane (Cat. No 10600002; GE Healthcare) using a Mini-Protean III Electrophoresis System (Bio-Rad). Gels and membranes were initially allowed to equilibrate separately in transfer buffer (100 ml 10 x TB (250 mM Tris Base, 1.92 M Glycine) 700 ml distilled H₂O and 200 ml methanol; pH 8.3) for 10 minutes, then sandwiched between Whatman filter paper (Cat. No LC2008; Invitrogen) and sponges (Cat. No EI9052; Invitrogen) and placed in the tank submerged in 1X transfer buffer. Transfer of proteins was run at 100V for 2 hours at 4°C. Following transfer, nitrocellulose membranes were washed in distilled H₂O, and then stained with Ponceau (0.1% (w/v) Ponceau S Red powder (Cat. No P3504; Sigma-Aldrich) in 5% (v/v) acetic acid (Cat. No 30010.292; VWR) for 5 minutes, to check for complete and even transfer. Ponceau staining was washed off with distilled H₂O. Non-specific binding was prevented by blocking the membrane in 5% BSA or 5% skimmed milk powder (Cat. No LP0031; Oxoid) in Tris-buffered saline (TBS; 20 mM Tris Base, 137 mM NaCl; pH 7.6) with 1% Tween-20 (v/v) (TBST) for 1 hour. Membranes were incubated overnight at 4°C in primary antibody in 1% BSA in TBST, details of antibodies can be found in Table 2.4. Excess primary antibody was removed by washing in TBST (3 x 5 minutes), membranes were then incubated in goat anti-rabbit horse radish peroxidase (HRP) conjugated secondary antibody (Cat. No P0448; Dako) in 1% BSA for 1.5 hours at room temperature. Following further washing in TBST (3 x 10 minutes), protein bands were visualised using enhanced chemiluminescence (ECL) by adding
Chapter 2: Materials and methods

peroxidase substrate (Cat. No 32209 Thermo Scientific) and exposing the membrane to high performance chemiluminescence film (Cat. No 28906837; GE Healthcare), or through digital imaging (ImageQuant LAS 4000; GE Healthcare). Protein bands were quantified using Image Studio Lite software (v5.25; LI-CORE Biosciences). Signal was normalised first to experimental control and then loading control (either Calnexin or α-tubulin).

Table 2.4 Primary antibodies for western blotting

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Species</th>
<th>Optimal concentration (µg/ml)</th>
<th>Catalog number; Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>Rabbit mAb</td>
<td>0.017</td>
<td>#4267; Cell Signaling Technology</td>
</tr>
<tr>
<td>ErbB2</td>
<td>Rabbit mAb</td>
<td>0.022</td>
<td>#4290; Cell Signaling Technology</td>
</tr>
<tr>
<td>ErbB3</td>
<td>Rabbit mAb</td>
<td>0.025</td>
<td>#12708, Cell Signaling Technology</td>
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<tr>
<td>Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)</td>
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<td>0.5</td>
<td>#4370, Cell Signaling Technology</td>
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<tr>
<td>Phospho-AKT (Ser473)</td>
<td>Rabbit mAb</td>
<td>0.086</td>
<td>#4060, Cell Signaling Technology</td>
</tr>
<tr>
<td>Calnexin</td>
<td>Rabbit pAb</td>
<td>0.2</td>
<td>#ab22595, Abcam</td>
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<tr>
<td>α-tubulin</td>
<td>Rabbit mAb</td>
<td>0.003</td>
<td>#2125, Cell Signaling Technology</td>
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2.4.4 Haematoxylin and eosin staining

Sections were initially dewaxed in Histoclear (2 x 5 minutes) then rehydrated in decreasing concentrations of absolute ethanol (100%, 95%, 70% for 1 minutes each) and washed in distilled H₂O. Nuclei were stained using Harris Haemotoxylin (Cat. No; LAMB/230-D, Thermo Scientific) for 10 minutes, then washed in running tap water until water ran clear. Acid Alcohol (1%, Cat. No 3803651E; Surgipath) was used to leach out excess Haemotoxylin staining before sections were held in blueing reagent, Scotts tap water solution (Cat. No 3802901E; Surgipath), for 2 minutes. Cytoplasm was stained using Eosin Y (Cat. No 6766009; Thermo Scientific) for 2 minutes then sections were washed once again in tap water until water ran clear. Finally, sections were dehydrated in increasing ethanol concentrations (70%, 95%, 100% for 20 seconds, 30 seconds and
1 minute respectively), then incubated with Histoclear (2 x 5 minutes) and mounted with coverslips in DPX mounting medium (Cat. No 360294H; VWR).

2.4.5 Immunofluorescence

2.4.5.1 Immunofluorescence with paraffin embedded tissue

Sectioned tissue was dewaxed in Histoclear (2 x 5 minutes) and rehydrated through decreasing concentrations of ethanol (100%, 95%, 70% for 5 minutes each), then washed in distilled H\textsubscript{2}O. To unmask epitopes, antigen retrieval was performed either with citrate buffer (pH 6.0, 0.34g citric acid anhydrous (Cat. No C/6240/53; Fisher Scientific), 2.47g tri-sodium citrate (Cat. No S/3320/52; Fisher Scientific) into 1L H\textsubscript{2}O) or Tris buffer (pH 10.0, 12.11g Tris base (Cat. No 103156X; VWR), 50g Urea (Cat. No 28877.292; VWR) in 1L H\textsubscript{2}O). Sections were boiled with antigen retrieval buffer for 10 - 20 minutes and left until cool before washing in PBS (2 x 5 minutes). Non-specific binding was blocked by incubation with 20% (v/v) species-specific serum supplemented with 4% (w/v) BSA in PBS for 30 minutes. Depending on the species in which the secondary antibody was raised, matching serum was used, either normal goat serum (NGS; Cat. No 120316; SAFC Biosciences) or normal donkey serum (NDS; Cat. No D9663; Sigma-Aldrich). Slides were then incubated overnight at 4°C with the primary antibody or non-immune immunoglobulin (IgG) isotype control diluted in 2% (v/v) serum. Details of primary antibodies are shown in Table 2.5, and IgG controls in Table 2.6. Following an overnight incubation, slides were washed in PBS (3 x 10 minutes) to remove excess primary antibody. An additional step was included for slides stained for apoptosis markers, sections were double labelled with terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL; Enzyme: Cat. No 11767305001, Label: Cat. No 11767291910, Roche) in a 1:9 ratio (Enzyme : Label) for 60 minutes at 37°C, then washed 3 times for 10 minutes in PBS. All slides were then incubated with appropriate Alexa Fluor conjugated secondary antibody, either Alexa488, 555 or 633 diluted in PBS for 60 minutes at room temperature, (details of secondary antibodies in Table 2.7). Excess secondary antibody was removed with PBS washes (2 x 5 minutes) before slides were briefly counterstained with 1 μg/ml 4’,6-diamidino-2-phenylindole (DAPI, Cat. No D8417, Sigma-Aldrich) for 3 minutes. All sections were then mounted with coverslips in Prolong Gold antifade reagent.
containing DAPI (Cat. No P36931; Invitrogen). Fluorescently stained sections were imaged using a confocal laser-scanning microscope.

### 2.4.5.2 Immunofluorescence for cultured cells

Antigen retrieval was performed on fixed cells adhered to coverslips by submersion in pre-chilled methanol (Cat. No 20847.307; VWR) and acetone (Cat. No 100034Q; VWR) for 3 minutes and 1 minute respectively. Cells were blocked in 5% (v/v) normal goat serum in PBS for 20 minutes to prevent nonspecific binding. Coverslips were exposed to primary antibodies, detailed in Table 2.5 or IgG isotype control, detailed in Table 2.6, overnight at 4°C on a rocking platform. Washing in PBS (3 x 5 minutes) was performed the following day to removed excess primary antibody before incubation with appropriate secondary antibody (Table 2.7) for 1 hour at room temperature. Coverslips were washed briefly in PBS (2 x 5 minutes), then counterstained with DAPI for 3 minutes before mounting onto Superfrost glass slides using Prolong Gold antifade reagent. Fluorescent staining was imaged using a confocal laser-scanning microscope.

#### Table 2.5 Primary antibodies for immunofluorescence

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<tr>
<th>Primary Antibody</th>
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<th>Antigen retrieval buffer</th>
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<td>#9664; Cell Signaling Technology</td>
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<td>0.365</td>
<td>#C6219; Sigma-Aldrich</td>
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<tr>
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<td>Rabbit mAb</td>
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<td>0.055</td>
<td>#4290; Cell Signaling Technology</td>
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<tr>
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<td>Rabbit mAb</td>
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<td>0.84</td>
<td>#12202; Cell Signaling Technology</td>
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<td>Vasa (DDX4)</td>
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<td>Citrate</td>
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Table 2.6 Non-immune immunoglobulin (IgG) isotype controls for immunofluorescence

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<td>Rabbit IgG</td>
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<td>Mouse IgG1</td>
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Table 2.7 Secondary antibodies for immunofluorescence

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<td>#A-11008; Invitrogen</td>
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<tr>
<td>Alexa Fluor488</td>
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<td>Goat anti-rabbit IgG</td>
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<td># A-21428; Invitrogen</td>
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<td>Goat anti-rabbit IgG</td>
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2.4.5.3 Quantification of immunofluorescence

All slides in the same experiment underwent immunofluorescence labelling simultaneously, and during the acquisition of images on a confocal laser-scanning microscope, the gain and offset settings were kept fixed across all samples. To analyse differential staining in cultured preantral follicles and ovaries, images were opened in ImageJ, the Red-Green-Blue (RGB) channels were separated into individual grey scale images using RGB stack command, then staining intensity in the channel of interest (red or green depending on the secondary antibody used) was thresholded. Thresholding divided pixels into those with a signal greater than a fixed cut off and those below. The threshold was fixed by comparing pixels over the threshold with the RGB image, so that the pixels above the threshold reflected the staining seen in the image. The established threshold was kept consistent across all slides analysed. Pixels above the fixed threshold were measured as a proportion of total GC area in cultured follicles, or total
ovary area. Alternatively, staining for Ki67, cleaved caspase-3 or TUNEL in cultured preantral follicles was analysed by counting GC nuclei stained positively for cell proliferation and apoptosis markers as a proportion from the total number of GCs i.e. the total number of DAPI stained nuclei.

To determine proportion of nuclear staining, images were RGB stacked on ImageJ and the nuclear DAPI (blue) channel isolated. A threshold was set for DAPI staining and nuclei were outlined using ‘Analyse Particles’ function. The outlined particles were overlaid on the channel with the desired immunofluorescence staining. Staining above an established threshold within the DAPI overlaid particles was measured as well as total staining. Percentage staining co-localised within DAPI particles was then calculated. A more detailed description of this protocol can be found in Section 6.2.5.

2.4.6 Morphological analysis

2.4.6.1 Cultured mouse follicles: growth analysis

Photographs of individual follicles were taken at the start of the culture period and every 24 hours using a Nikon digital camera DXM 1200 attached to a Nikon Eclipse TE300 light microscope using a x20 objective. Morphological characteristics were used to assess follicular health at the end of culture (72 hours). Follicles were excluded from further analysis if (i) the oocyte was not centrally located, (ii) the oocyte had been extruded, (iii) the oocyte was misshapen or had darkened or (iv) the GCs had darkened indicative of atresia. All images were imported in a custom made database (FileMaker Pro 11.v2; http://www.filemaker.com) as previously described (Laird et al., 2017) to allow consistent exclusion of follicles which were classed morphologically unhealthy. Follicle area was measured using ImageJ 1.45s (https://imagej.nih.gov/ij/). All measurements were completed blind as to treatment.

2.4.6.2 Cultured mouse ovaries: follicle activation analysis

Cultured ovaries were fixed, embedded, sectioned and labelled for immunofluorescence detection of germ cell specific vasa (DDX4) protein (see Section 2.4.5.1). Two central sections from each ovary, at last 25 µm apart were chosen for morphological analysis. Imaging of whole ovary sections was completed by tiling multiple images (x40) on a confocal laser-scanning microscope (Leica SP5). Only vasa-stained oocytes with a clear
nucleus were included and area measured on ImageJ. All analysis was completed blinded in regard to treatment. Oocyte area was used as a surrogate marker for follicle activation; see Section 5.1 for further details. Work undertaken by Elizabeth Oliver (PhD thesis, 2017) found that a cut off of 275 µm² yielding comparable results to morphological follicle classification (based on GC number, morphology and degree of multi-layering), so was chosen as an appropriate threshold for all further morphological analysis. Follicles with an oocyte larger than 275 µm² were classified as growing, and all follicles with oocytes measuring less than 275 µm² were classified as primordial.

2.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism (v5 and v7; GraphPad Inc). All data were assessed for normal distribution using D'Agostino & Pearson omnibus normality test. Normally distributed data were analysed by unpaired t test or analysis of variance (ANOVA). Data not normally distributed were analysed by the non-parametric Kruskal-Wallis or Mann-Whitney test. Post-hoc tests were used where appropriate. The exact statistical tests are named in each figure legend, where n denotes experimental biological repeats. In all cases, a P value less than 0.05 was considered statistically significant.
Chapter 3 ErbB Receptor and EGF-like ligand expression in mouse ovarian tissue

3.1 Introduction
The essential role for the EGF family during ovulation has led to their thorough characterisation in large antral follicles in the adult ovary, however little work has been performed investigating ErbB expression at the preantral stages of follicle development. The EGF-like ligands Areg, Ereg and Btc have been localised to the GCs of the pre-ovulatory follicle, responsible for relaying the LH signal to the LHR-devoid cumulus oocyte complex. Localisation of these gene products through in situ hybridisation showed that expression of the Areg, Ereg and Btc was restricted to the mural GCs at the periphery of the follicle, and with the exception of Btc, there was no specific signal detected in the cumulus GCs or the oocyte itself (Park et al., 2004). EGFR has been detected in the mural and cumulus GCs of the preovulatory follicle. A surge in LH promotes up-regulation of the EGF-like ligands which can signal either within the mural layer or diffuse across the antrum to stimulate EGFR in the cumulus GCs, which triggers oocyte maturation and cumulus expansion (Conti et al., 2006). GC-specific EGFR knock out mice display impaired oocyte meiotic resumption and cumulus expansion (Hsieh et al., 2011). Although the spatiotemporal expression of EGFR and EGF-like ligands, Areg, Ereg and Btc, has been well characterised in the pre-ovulatory follicle, the developmental stage at which the follicle starts to transcribe and translate EGFR and EGF-like ligand protein is unknown. This chapter aimed to investigate the presence of EGFR and EGF-like ligands during the earlier stages of follicle development. Additionally, the presence of the other ErbB receptor subtypes and EGF-like ligands were investigated which have been overlooked in the research to date.

Earlier work from our lab identified Egfr, Erbb2 and Erbb3 mRNA in the adult and d16 mouse ovary, as well as Egfr and Erbb2 mRNA in isolated preantral follicles and GCs (Figure 3.1, courtesy of Victoria Atess, PhD Thesis, 2015). This chapter contains the continuation of this work, with the aim to dissect the relative expression levels of ErbB subtype mRNA present in preantral follicles and investigate ErbB protein expression.
Chapter 3: ErbB Receptor and EGF-like ligand expression in mouse ovarian tissue

### 3.1.1 Specific aims

- Investigate the relative expression of ErbB receptor mRNA in isolated preantral follicles.
- Examine protein expression of ErbB receptors in mouse ovarian tissue of differing developmental stages.
- Identify EGF-like ligands present in mouse ovarian tissue.

### 3.2 Results

#### 3.2.1 Quantification of ErbB receptor mRNA in mouse preantral follicles

The preliminary RT-PCR screen demonstrated the presence of *Egfr* and *Erbb2* mRNA as well as faint *Erbb3* mRNA in isolated preantral follicles (Figure 3.1). In order to investigate the relative levels of the ErbB subtypes in isolated follicles, absolute transcript abundance of ErbB mRNA was quantified using quantitative RT-PCR (Figure 3.2). Preantral follicles (approx. 75 - 130 µm diameter) were isolated and pooled from d16 mice, quantitative RT-PCR was performed and Ct values were compared against standard curve values from positive control tissue to derive absolute mRNA amount. Consistent with the RT-PCR screen, *Egfr* and *Erbb2* were the most highly expressed transcripts, with an average of 0.0044 fg and 0.0066 fg per follicle respectively. *Erbb3* mRNA was present but detectable at very low levels in follicles. *Erbb4* mRNA was undetectable in all samples analysed.
Chapter 3: ErbB Receptor and EGF-like ligand expression in mouse ovarian tissue

3.2.2 Protein expression of ErbB receptors in mouse ovarian tissue

Following the results seen in gene expression, the expression of ErbB protein in the mouse ovary was investigated. Western blotting was used to detect protein expression of subtypes EGFR, ERBB2 and ERBB3, as these were the three ErbBs present as mRNA. EGFR protein was expressed in both adult and juvenile d16 whole ovary, but interestingly not in the d4 ovary (Figure 3.3A). ERBB2 protein was expressed in all whole ovary samples, with strongest expression at d16. ERBB3 protein was absent from all mouse ovarian tissue examined, however a very faint band was observed in one of the adult ovary lysate samples as shown in Figure 3.3B. The faint band was detected at the predicted molecular weight of 185kDa.

In order to assess the degree of protein expression attributable to the preantral follicle within the whole ovary, follicles were mechanically isolated and cleaned of surrounding tissue from d16 ovaries. Additionally, GCs were isolated from antral follicles of d26 mouse ovaries and oocytes were isolated from d16 ovaries, to investigate cell-specific expression. To accurately quantify expression levels across the differing tissue types, 5µg of total protein lysate was loaded into each western blot. However, the quantity of
protein collected from isolated oocytes was significantly lower than other samples. This was due to the technical constraints of cleanly isolating sufficient intact oocytes with minimal GC contamination. Despite the lack of signal of ErbB expression in oocytes, it therefore cannot be definitively concluded that ErbB protein was not present in oocytes, and further investigation is needed. Both EGFR and ERBB2 protein were expressed in isolated preantral follicles and isolated GCs (Figure 3.3A).

**Figure 3.3 Western blot screen showing presence or absence of ErbB receptor protein (ErbB1-3).**

(A) Protein extracted from adult ovary (adult), d16 ovary (d16), d4 ovary (d4), pooled preantral follicles (Fol), pooled oocytes (Oo), antral granulosa cells (GC), mouse liver used as positive control for EGFR protein, and mouse lung for ErbB2 and ErbB3 protein (+ve). 5 µg of protein was loaded into each sample, except isolated oocytes. Calnexin was used as an internal loading control. (B) Molecular weight markers shown against EGFR, ERBB2 and ERBB3 protein expression in adult ovary.

The protein expression profiles give an indication of ErbB expression in progressing follicle stages. To examine the composition of the ovary at the developmental days in the western blot screen, fresh ovaries from adult, d16 and d4 mice, as well as isolated follicles were fixed, sectioned and H & E stained. The d4 ovary is made up of primarily dormant primordial follicles with few activated transitional and primary follicles, while the older d16 ovary contains many more primary and secondary follicles with the emergence of large multi-layered preantral follicles (Figure 3.4). Adult ovaries contain all of the earlier follicle stages mentioned as well as large antral follicles, pre-ovulatory follicles and CLs.
Figure 3.4 Mouse ovary structure at differing ages.
Ovaries were collected from adult (A), d16 (B) and d4 (C) mice for the western blot screen. Representative images stained with H&E are shown of a typical ovary at each of these ages. Preantral follicles (D) were also isolated from d16 ovaries. Scale bars are ovaries (A-C) = 200 µm and isolated follicle (D) = 50 µm.

Calnexin, an integral protein of the endoplasmic reticulum, was used as an internal loading control and relative ErbB protein expression was quantified across all samples (Figure 3.5). Quantification confirmed the results seen visually, with EGFR expressed highly in the adult and d16 ovary and virtually absent from the d4 ovary. Interestingly, there was significantly more EGFR protein in the whole d16 ovary than the preantral follicles isolated from the same age, suggesting EGFR is also present in the cell types surrounding the developing follicles, such as thecal and stromal cells. Protein quantification confirmed that ERBB2 protein was weakly expressed in the adult and d4 whole ovary, peaking at d16. Analysis revealed a trend toward higher ERBB2 expression in isolated follicles than whole d16 ovary, indicating the preantral follicle, in particular the GCs, are the principal source of ERBB2 protein. ERBB3 protein was only detectable in one adult ovary sample and therefore could not be quantified in this study.
Figure 3.5 Quantification of EGFR and ERBB2 protein expression in mouse ovarian tissue.

Protein lysates extracted from adult ovary (adult), d16 ovary (d16), d4 ovary (d4), pooled preantral follicles (Fol), and pooled granulosa cells (GC). Signal intensity calculated relative to calnexin as internal loading control, and justified to the adult ovary expression. Values are mean +/- SEM (n = 2 - 4 ovaries), statistically analysed by one way ANOVA with Tukey’s multiple comparison test, where * p < 0.05, ** p < 0.01.

Immunolocalisation of EGFR protein in the ovary has been attempted by our research group and others however, no appropriate commercially available antibody for immunohistochemical detection of EGFR has been found. Work is on-going into visualising EGFR protein in the ovary. Previous work performed in the group immunolocalised ErbB2 protein in whole ovary sections (Victoria Atess, PhD thesis, 2015). Images shown in Figure 3.6 are all courtesy of Victoria Atess. ErbB2 protein was absent from non-growing primordial follicles, and was first detected in the GC cell membranes from the transitional stage onward (Figure 3.6A-B). ErbB2 protein was visible in primary and multi-layered follicles (Figure 3.6C-D), but absent from larger antral follicles (Figure 3.6E). Atess quantified the intensity of ErbB2 immunolabeling in different stage follicles. Quantification of ErbB2 expression confirmed results seen visually. ErbB2 intensity significantly increased from the primordial to primary...
transition, peaking in the primary follicle, then declined as the number of GC layers increased (Figure 3.6F).

**Figure 3.6 ErbB2 protein immunolocalisation in mouse ovarian tissue.**
Nuclei are labelled with DAPI (blue). ErbB2 staining (green) in A) primordial follicles, B) transitional follicles, C-D) growing preantral follicles and E) antral follicles. Scale bars = 10 µm (A, B, D); 20 µm (C) and 50 µm (E). Strongest expression seen between GC membranes indicated by white arrowheads, weaker expression seen between oocyte and GCs indicated by black arrowheads, white arrows indicate expression in transzonal processes. F) Quantification of ErbB2 at progressive follicle stages. Values shown are individual data points and mean (n = 9 d16 ovaries) statistically analysed using Kruskal Wallis with a Dunn’s multiple comparison test, where ** p < 0.01, *** p < 0.001, **** p < 0.0001. p: primordial, t: transitional, 1°: primary, 1°+: primary plus, 2°: secondary, ML: multi-layered, An: antral follicle, dotted line: basal lamina. Courtesy of Victoria Atess, PhD thesis, 2015.

**3.2.3 EGF-like ligand expression in mouse ovarian tissue**
The two principal ErbB subtypes expressed in mouse ovary as both mRNA and protein were EGFR and ErbB2. ErbB2 has no known binding ligand but has the ability to form potent heterodimers with the EGFR (Yarden and Sliwkowski, 2001). The EGFR can be bound and activated by the EGF family of growth factor ligands including EGF, TGF-α,
HB-EGF, Areg, Btc, Ereg and Epgn (Harris et al., 2003), which all share an EGF-like motif in their extracellular domain (Schneider and Wolf, 2009). For this reason, these seven ligands were examined in an RT-PCR screen of mouse ovarian tissue to establish their presence (Figure 3.7). Another class of ligands, the neuregulins, were not included in this screen as they primarily bind and activate both ErbB3 and ErbB4 (Falls, 2003), which were shown to be largely absent in mouse ovarian tissue.

Transcripts for Tgfa, Hbegf, Egf, Btc, Ereg and Areg were all present in adult and juvenile d16 whole ovary as well as isolated preantral follicles. Tgfa and Egf transcripts were detected both in GCs and isolated oocytes. In contrast, Hbegf, Btc, Ereg and Areg mRNA was present exclusively in the isolated GCs. Epgn was undetectable in all mouse ovarian tissue examined.

The EGF-like ligands expressed in preantral follicles were then further examined to reveal the relative transcript abundance within preantral follicles using quantitative RT-PCR (Figure 3.8). Tgfa was the most highly expressed transcript with on average 0.0024 fg per follicle. Hbegf, Egf and Btc were all expressed at a similar level in preantral
follicles. *Ereg* and *Areg* were both detectable at very low levels, while *Epgn* was undetectable.

\[ \text{Figure 3.8 Quantification of EGF-like ligand mRNA in isolated preantral follicles.} \]

EGF-like ligand transcripts ordered by abundance calculated by absolute mRNA quantification in isolated preantral follicles. Values are mean ± SEM (n = 6 ovaries, 20 isolated follicles used per ovary).

### 3.3 Discussion

#### 3.3.1 ErbBs are expressed in preantral follicles

Interest in the spatiotemporal expression of the ErbB receptors to date has concentrated on the post-pubertal ovary, localising EGFR to the mural and cumulus GCs of large antral follicles (Garnett et al., 2002, Gall et al., 2004). This study found that ErbB mRNA and protein expression was detected in the pre-pubertal mouse ovary weeks before cycling initiates.

The western blot profiles give an indication of ErbB protein expression at progressing follicular stages. As EGFR protein is almost undetectable in the d4 ovary, it could be extrapolated that EGFR protein is likely absent from primordial follicles. EGFR is present in the d16 ovary, suggesting that EGFR protein is translated following follicle activation and may play a role in preantral follicle development rather than activation. This hypothesis is addressed later in Chapters 4 and 5. EGFR protein was detected in adult ovary, unsurprisingly as others have shown that its presence is essential for
cumulus expansion and ovulation (Conti et al., 2006). In comparison, ERBB2 protein is fainter in the adult and d4 ovary peaking at d16 when the ovary contains largely preantral follicles. The peak in ERBB2 protein at this age could point to a role in preantral follicle development, explored further in Chapter 4. However, it is important to note that various other structures make up the composition of the ovary, such as the supporting stroma, which may also contribute to the ErbB protein expression.

3.3.2 EGFR is expressed in GCs of growing preantral follicles

EGFR protein was detected by western blotting in isolated follicles and GCs, however work is on-going to immunolocalise EGFR protein to examine stage- and cell- specific expression in the mouse ovary. Studies published using other rodent species may provide some insights into the predicted spatiotemporal expression of the EGFR. Early studies using radioautography of the rat ovary showed that EGF binding sites, indicating the presence of EGFR, were present in the ovary but restricted to larger growing follicles (Chabot et al., 1986). Adult rats were intravenously injected with $^{125}\text{I}$ EGF and binding sites were detected by light microscope radioautography, displaying distinct labelling in the theca interna cells of large preantral, pre-ovulatory and atretic follicles as well as in the luteal cells of CLs. Interestingly, there was no significant labelling detected in primordial or primary follicles, consistent with the lack of EGFR protein observed in the d4 mouse ovary in the present study. In vitro studies, using topical localisation of $^{125}\text{I}$ EGF on ovarian sections, confirmed the observations seen in vivo in the rat ovary and revealed additional radioautographic labelling specifically within the GCs of growing and pre-ovulatory follicles. This is consistent with EGFR protein detected in mouse GCs in the present study. The lack of detection of EGF binding sites in the early rat preantral follicles may be due to the limited resolution of the radioautographs.

A further study in the hamster ovary investigated spatiotemporal expression of the EGFR during the oestrous cycle through immunofluorescence (Garnett et al., 2002). GCs from antral follicles and surrounding theca and stromal cells displayed strong EGFR immunoreactivity, with expression peaking in the mural GCs following the gonadotropin surge. GCs from primordial and preantral follicles also displayed EGFR immunoreactivity, but expression was relatively lower. In situ hybridisation revealed
that EGFR mRNA levels were consistent with the profile seen in protein expression following the same follicle stage-specific expression, peaking in the later stage follicle. Garnett et al., did observe strong immunofluorescence staining within the interstitial cells of small developing follicles which may explain the increased expression observed in whole d16 ovary compared to isolated follicles alone in the present study.

Although there appears to be some discrepancies regarding the follicle stage at which EGFR is first expressed, with some papers reporting expression in primordial follicles in the rodent (Garnett et al., 2002) and goat ovary (Silva et al., 2006), and others only within growing follicles (Chabot et al., 1986), there is a general consensus that the EGFR increases with follicle progression. There is strong evidence that EGFR expression is regulated by gonadotropin signalling (Fujinaga et al., 1994). FSH has been shown to significantly stimulate EGFR expression in rodent GCs (Garnett et al., 2002). As the preantral follicle develops it becomes increasingly responsive to FSH (Hardy et al., 2017), which may provide an explanation for the increase of Egfr mRNA and EGFR protein observed in the developing follicle. Additionally, mice carrying a deletion in the gene encoding the β-subunit of FSH, have shown that the stage-specific increase in EGFR may be dependent on FSH signalling (El-Hayek et al., 2014). Fshb+/- mice displayed a significant increase in Egfr mRNA and EGFR protein in the ovary between d12 and d21 coinciding with the emergence of antral follicles, whereas Fshb-/- mice while they expressed the same level of Egfr and EGFR at d12, failed to accumulate EGFR normally by d21, resulting in approximately half as much EGFR expression as the Fshb+/- mice.

To dissect the cell-specific expression of EGFR in isolated follicles, mRNA and protein was extracted from denuded oocytes and isolated GCs. Egfr mRNA and EGFR protein were present in isolated GCs. Egfr mRNA was absent from isolated oocytes but due to the technical constraints of collecting sufficient protein from oocytes it cannot yet be concluded that EGFR protein is absent from oocytes in the mouse. Interestingly, EGFR protein expression has been observed on the surface of oocytes in the hamster (Garnett et al., 2002) and goat (Silva et al., 2006) ovary regardless of follicle stage. EGF-like peptides induce oocyte maturation in vitro so it may be expected that the EGFR is present within the oocyte of developing follicles (Park et al., 2004). However, one study showed that EGF-like ligands in vitro stimulate meiotic resumption, as marked by GVBD,
in cumulus cell-enclosed oocytes but not in denuded oocytes, which remained in meiotic arrest (Downs and Chen, 2008). This strongly suggests that cumulus cells are required to receive and respond to the EGFR signal. Additionally, the presence of the EGFR inhibitor, AG1478, or the gap junction inhibitor, glycrrhetinic acid, blocked meiotic resumption in cumulus cell-enclosed oocytes but not in denuded oocytes, suggesting EGFR is expressed in the cumulus cells and cell-cell signalling between cumulus cells and oocyte in a gap junction-dependent process is needed to relay the EGFR signal. These results support our findings that EGFR is expressed primarily in the GCs.

3.3.3 ErbB2 expression peaks in preantral follicles

There is limited published data regarding ErbB2 expression in the juvenile mouse ovary. In the present study, Erbb2 mRNA and ERBB2 protein was detected in all ages of ovary examined, present in the neonatal d4 and peaking in the d16 ovary, with the emergence of primary and secondary follicles with some larger multi-layered preantral follicles. Previous work from the group, conducted by Victoria Atess, revealed follicle stage specific immunolocalisation of ErbB2 protein in mouse ovaries. ErbB2 was first observed at the interface of GCs from the transitional stage onward, peaked in the GCs of growing preantral follicles and became weaker as the follicle reached the antral stage. These results suggest that ErbB2 is playing a role during preantral follicle development. As ErbB2 first appears at the interface of GCs in the transitional follicle, this may indicate its involvement in GC cuboidalisation. During primordial follicle activation, adherens junctions such as N-cadherin and nectin-2 also appear at the elongating membrane of GCs (see Figure 1.2) (Mora et al., 2012). ErbB2 may regulate or be regulated by these adherens junctions as they display strikingly similar spatiotemporal expression.

There has been little research into ErbB2 expression in noncancerous ovarian tissue, however one study detected Erbb2 mRNA by in situ hybridisation in the ooplasm of primordial follicles from d2 postnatal rat ovaries (Li-Ping et al., 2010). Although this is conflicting with our immunofluorescence findings in the primordial follicle, the authors did observe that Erbb2 levels increased in follicles that had initiated growth. This conflicting result may be explained by species variability or it may suggest that ErbB2 is transcribed but not translated to protein in the primordial follicle. This study also
detected ERBB2 protein expression by western blotting in neonatal rat ovaries, supporting our findings.

### 3.3.4 ErbB3 was undetectable in preantral follicles

*Erbb3* mRNA was detected weakly in the whole ovary and isolated preantral follicle cDNA, and ERBB3 protein was expressed weakly in one adult ovary sample collected. There is limited research regarding ErbB3 in the developing ovary, however ErbB2, which is highly expressed in preantral follicles, is known to be the preferential dimerisation partner of ErbB3, forming the most potent of the ErbB dimers (GrausPorta et al., 1997). ErbB3 can directly activate the PI3K pathway, through phosphotyrosine binding sites unlike EGFR (Elenius, 1999), which is important in follicle activation, growth and survival (Makker et al., 2014). ErbB3 may, if present, provide a mechanism to diversify signalling by ErbB2 in preantral follicles. Additionally, ErbB3 binds different ligands to the EGFR, namely NRG1 and NRG2 (Falls, 2003), which may further expand the role of ErbBs in preantral follicles. NRG1 and ErbB3 have both been implicated in primordial follicle formation and colonisation of the genital ridge by PGCs (Kierszenbaum and Tres, 2001), however their presence and role in later follicle development is unclear. NRG1 mRNA expression has been shown to change during primordial to primary follicle transition through microarray analysis in rat ovaries (Kezele et al., 2005b). One study investigated the role of NRG further in the rat ovary. Neonatal ovaries cultured for 14 days with NRG displayed no change in follicle development, with no difference observed in the percentage of developing follicles at the end of culture (Nilsson et al., 2006), which doesn't support a role for NRG in regulating follicle activation. The limited expression of ERBB3 protein detected in whole ovary may indicate its lack of biologically relevance in the mouse ovary, however its presence should be acknowledged.

### 3.3.5 Possible ErbB dimer combinations

The two ErbB subtypes expressed highly in the mouse ovary were EGFR and ErbB2. Therefore, following ligand binding there are two probable ErbB dimer combinations; EGFR homodimers and EGFR-ErbB2 heterodimers. Upon EGF-like ligand stimulation, associations between EGFR and ErbB2 at the cell surface have been described in EGFR and ErbB2 transfected C2C12 cell lines (Wehrman et al., 2006). It has been suggested
that the presence of ErbB2 protein in target tissue results in ErbB2-containing heterodimers forming preferentially over other dimer combinations (GrausPorta et al., 1997, Tzahar et al., 1996). However, quantitative studies at the cell surface in C2C12 cells afore mentioned demonstrate that EGFR-ErbB2 heterodimers form with a similar but not greater affinity than EGFR homodimers in response to EGF stimulation (Wehrman et al., 2006). ErbB2-containing heterodimers are believed to be the most potent dimer combination complexes (PinkasKramarski et al., 1996), as ErbB2 can potentiate and prolong signal transduction pathways, increase ligand affinity, relax ligand specificity and alter receptor trafficking (GrausPorta et al., 1997), in contrast EGFR homodimers signal only weakly (Yarden and Sliwkowski, 2001). The relative expression profiles of the ErbB subtypes across the varying aged ovaries may suggest different dimer combinations forming preferentially at different stages of follicle development. An array of signalling cascades can be activated downstream of the ErbB receptors dependent on dimer composition meaning the outputs are wide ranging, implicated in cell division, migration, adhesion, differentiation and apoptosis. Varying dimer composition may provide a mechanism by which EGF signalling could control proliferation as well as differentiation in the developing follicle.

### 3.3.6 EGF-like ligands are expressed locally in the mouse ovary

Having shown that EGFR and ErbB2 are highly expressed in preantral follicles, it was investigated if local ligands that bind to ErbBs are expressed in the ovary, especially as early preantral follicles are thought to rely predominantly on local intraovarian regulators. EGF-like ligands Tgfa, Hbegf, Egf, Btc, Ereg and Areg were all expressed in preantral follicles, with Tgfa, Hbegf, Egf and Btc the most abundant.

TGF-α is produced locally in the human ovary, and a number of studies have detected the protein in follicular fluid samples from patients undergoing IVF programmes. One study found that TGF-α was present in 90% of all patient samples collected (McWilliam et al., 1995). Within the ovary, theca cells appear to be the primary source of TGF-α synthesis. TGFA mRNA has been found consistently in the theca cells of antral follicles in the human ovary (Lobb, 2009, Tamura et al., 1995), as has TGF-α protein, with strong immunolocalisation in the theca cells of growing follicles (Reeka et al., 1998, Qu et al., 2000b). Additionally, TGF-α has been immunolocalised to the theca-interstitial cells.
within the rat ovary (Kudlow et al., 1987). Evidence for TGF-α synthesis in the oocyte and GCs however is inconsistent. RT-PCR screening found that TGFA mRNA was undetectable in human GCs of all follicle sizes (Lobb, 2009). However, earlier studies in the human ovary found along with strong staining for TGF-α in the thecal cells, TGF-α was also immunolocalised in the oocytes of primordial and early preantral follicles, as well as the GCs of preantral follicles (Reeka et al., 1998, Qu et al., 2000b). The present study found that Tgfa mRNA was present in the whole ovary but also in isolated preantral follicles. There is a possibility that there may be some minor theca cell contamination when isolating preantral follicles from surrounding tissue, however, Tgfa was also detected in isolated GCs and oocytes. Future experiments should investigate TGF-α immunolocalisation in the juvenile mouse ovary to confirm the primary source of TGF-α. These results suggest that Tgfa produced in the oocyte, GCs or theca cells can signal locally through the EGFR expressed in the GCs to regulate mouse preantral follicle development. Similarly, bovine theca cells cultured in serum free medium secrete TGF-α which stimulates the growth of both granulosa and theca cells (Skinner and Coffey, 1988). This system demonstrates in other model species that TGF-α regulates follicle development through both paracrine and autocrine signalling to regulate proliferation.

EGF itself has been detected in human follicular fluid (Westergaard and Andersen, 1989) although there remains some debate over the source of this growth factor, whether it diffuses from the serum or is secreted locally from the ovary. Egf mRNA has been molecularly probed for but found absent from granulosa and theca cells in the bovine model (Skinner and Coffey, 1988) and both mRNA and EGF protein were not detectable in the human ovary by in situ hybridisation or immunolocalisation (Tamura et al., 1995). However, some localisation studies have detected weak immunostaining for EGF ligand in the oocytes and GCs of primordial and growing preantral follicles and in theca cells of larger antral follicles in the human ovary (Qu et al., 2000a, Reeka et al., 1998). Interestingly, the present study found Egf mRNA present in isolated preantral follicle, GCs and oocytes. This suggests, in the mouse at least, that EGF may also play a role in paracrine and autocrine signalling to regulate follicle development.

The EGF-like ligand Hbegf was also detected in preantral follicle in the present study, exclusively within the GCs. Although there is limited literature regarding HB-EGF in the
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ovary, HB-EGF has been observed in the adult rodent ovary previously in a cell- and stage-specific manner (Pan et al., 2004). *Hbegf* mRNA and the precursor form of HB-EGF protein was detected in the GCs of all early developing follicles, but absent from the pre-ovulatory follicle in the rat ovary. This is consistent with our findings and may place HB-EGF as another important local regulator of preantral follicle development.

*Areg, Ereg* and *Btc* have been thoroughly studied in the pre-ovulatory follicle, localised to the mural GCs (Park et al., 2004). However, it appears that *Btc* in particular is expressed earlier in the preantral stage follicles. Consistent with studies in preovulatory follicles, *Areg, Ereg*, and *Btc* were observed in the GCs rather than the oocyte of the follicle.

When considering expression of the EGF-like ligands it is also important to note that these factors are synthesised as inactive membrane precursors with a single membrane-spanning domain. The factors must be processed by proteolytic cleavage and shed as soluble mature peptides to be active. The proteolytic enzyme TACE/ADAM17 has been implicated in EGF factor processing in many types of tissue, and is expressed in cumulus GCs. TACE/ADAM17 activity is biologically important in the ovary, and it has been shown that if enzymatic activity is suppressed both oocyte maturation and cumulus expansion are disrupted (Yamashita et al., 2007). The expression of this protease is explored further in Chapter 4 in cultured preantral follicles. Further research should investigate the expression of EGF-like ligand protein in the mouse ovary as well as expression of the proteases that cleave them.
Figure 3.9 Hypothesised autocrine and paracrine signalling of EGF-like ligands and ErbB receptors in preantral follicles.

Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) are expressed in the oocyte and granulosa cells of isolated preantral follicles. While heparin-binding EGF (HB-EGF), betacellulin (Btc), amphiregulin (Areg) and epiregulin (Ereg) are produced by the GCs of preantral follicles. Local EGF-like ligands may signal through EGFR and ErbB2 receptors present in the GCs of neighbouring preantral follicles, or within the follicle itself.

3.4 Conclusions

In conclusion, mouse preantral follicles express EGFR and ErbB2 receptor subtypes as well as various EGF-like ligands, particularly TGF-α, EGF, HB-EGF and Btc. These results indicate that EGF-like ligands produced in the oocyte, GCs or theca cells may signal locally through ErbB receptors expressed in the preantral follicles itself or neighbouring preantral follicles to regulate development (Figure 3.9).
Chapter 4 Effect of EGF on preantral follicle development

4.1 Introduction

In light of the findings in Chapter 3, which confirmed the presence of the ErbB subtypes EGFR and ErbB2 in preantral follicles, investigation into the effect of stimulating and inhibiting these receptors and thus their importance in preantral follicular development was undertaken. The principal culture system used in this chapter is the individual follicle culture. Follicles were isolated intact and as enclosed units, meaning signalling loops between the GCs and oocytes were maintained, while signalling from the surrounding tissue was eliminated, except that from a limited number of theca cells (see Section 1.7.2). Individual cultures allowed for the accurate measurement of growth over the culture duration, and following culture healthy follicles were collected and taken for a range of analytical studies to uncover changes in gene and protein expression.

The EGF-like ligands present in preantral follicles, highlighted in Chapter 3, TGF-α, EGF, HB-EGF, Areg, Ereg and Btc, all share a structurally similar EGF module, a sequence containing six cysteines that is responsible for binding to the EGFR (Schneider and Wolf, 2009). All EGF module-containing proteins exhibit a high affinity for binding and activating the EGFR. The other ErbB subtype present, ErbB2, has no known binding ligands. Therefore, exogenous EGF was used as a prototypical ligand to simulate ErbB signalling in vitro.

Since the 1970s, it has been proposed that supplementing culture medium with EGF has beneficial effects on in vitro follicle and ovary cultures. Gospodarowicz and Bialecki in 1979 demonstrated increased GC replication with the addition of EGF to culture medium in rabbit, porcine, and human GC cultures (Gospodarowicz and Bialecki, 1979). Since then, exogenous EGF has been shown to stimulate follicle growth, maturation and survival in other model species (Celestino et al., 2011, Aguiar et al., 2017). This chapter contains a thorough and detailed analysis of the EGF network in mouse preantral follicles, not only investigating follicle growth and survival but also the signalling pathways and receptors involved in relaying the response to EGF as well as the
subsequent changes in gene and protein expression. One of the novel questions asked in this chapter is if EGF ligand is signalling through EGFR homodimers or EGFR-ErbB2 heterodimers. Additionally, the role of endogenous EGF-like ligands produced locally within the follicle unit was examined by employing various ErbB inhibitors alone and in combination with EGF. A summary of the ErbB pathway inhibitors used in this chapter is shown in Figure 4.1.

**Figure 4.1 ErbB pathway inhibitors used in preantral follicle culture.** The EGFR specific inhibitor AG1478 blocks kinase activity of the C-terminal tail. The ErbB2 inhibitor ii blocks kinase activity of ErbB2. U0126 is a specific MEK1/2 inhibitor blocking signalling through the MAPK cascade. Shc, Src homology 2; Grb2, growth factor receptor binding protein 2; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated protein kinases.
4.1.1 Specific aims

- Investigate the effect of EGF exposure on preantral follicle growth and GC proliferation and apoptosis in vitro.
- Dissect which ErbB receptors and signalling pathways are responsible for relaying EGF signalling in preantral follicles.
- Examine the effect of EGF on expression of genes involved in the regulation of follicle development in vitro.
- Examine the effect of EGF exposure on protein expression of key regulators of follicle development.

4.2 Results

4.2.1 EGF stimulates GC proliferation and preantral follicle growth

The effect of stimulating the ErbB receptors in preantral follicles was explored using EGF, a prototypical EGF-like ligand. Preantral follicles (75 - 130 μm diameter, 2-3 layers of GCs) were isolated from d16 mouse ovaries and cultured for 72 hours with and without exogenous EGF (10 ng/ml). Photographs were taken every 24 hours over the culture duration and total follicle area was measured using ImageJ (Figure 4.2A). Supplementation of culture medium with EGF increased preantral follicle growth significantly from 24 hours (Figure 4.2B). Follicle growth was expressed as a percentage relative to follicle area at start of culture (time 0 hour), to account for variability in starting follicle size. The largest increase in size was recorded at 24 hours with EGF, follicle growth then plateaued at 48 and 72 hours. During analysis, oocyte area was also measured and remaining GC area (total follicle area – oocyte area) was calculated (Figure 4.2C). It was revealed that the increase seen in total follicle area in the EGF exposed follicles was due to an expansion in the size of the GC compartment, with no change observed in oocyte size.
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Figure 4.2 EGF stimulates preantral follicle growth in vitro.
A) Representative images of follicles cultured in vehicle control or 10 ng/ml EGF, scale bar = 50 µm. B) Percentage change in follicle area relative to 0 hour is shown. Values are mean ± SEM, statistical analysis between treatments were made within each time point by unpaired t-test where *** p < 0.001 (n = 6 ovaries). C) Change in area of oocyte and granulosa cells (total follicle area - oocyte area) over culture period in follicles treated with EGF (10 ng/ml). Values are mean ± SEM. Numbers bracketed represent number of follicles in each treatment group.

The increase observed in GC area was explored further by culturing follicles for 24 and 72 hours, before fixing, paraffin-embedding, sectioning and immunostaining them for the proliferation marker Ki67 (Figure 4.3A). Ki67 is a nuclear protein necessary for cellular proliferation, and is present during all active phases of the cell cycle (G1, S, G2 and mitosis) but absent from resting cells (G0), making it an excellent marker for GC proliferation. Calculating the proportion of GC nuclei stained positively for Ki67 was used to assess GC mitotic activity. At 24 hours EGF treated follicles displayed a significantly higher proportion of positively stained nuclei for Ki67 than control follicles (Figure 4.3B). Interestingly, Ki67 staining was not increased at 72 hours, coinciding with the plateau seen in follicle growth at this time.
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Figure 4.3 EGF stimulates GC proliferation in preantral follicles in vitro.
A) Follicles cultured in vehicle control or EGF (10ng/ml) were fixed and stained for Ki67 (green) and counterstained with nuclei label DAPI (blue), representative images at 24 hours and 72 hours are shown, scale bar = 50 µm. B) Proportion of Ki67-positively stained nuclei in EGF treated follicles at 24 hours (n = 11 - 15 follicles) and 72 hours (n = 8 - 14 follicles). Bars shown are mean ± SEM, compared using unpaired t-test where **** p < 0.0001.

Follicles appeared morphologically healthy over the culture period with no obvious detrimental effect of EGF on health. Follicles were classed as unhealthy if the oocyte was not centrally located, was misshapen or it had been excluded, or if the GCs had darkened indicative of atresia. To investigate levels of GC apoptosis with EGF, follicles were double immunolabelled for apoptosis markers TUNEL and cleaved caspase-3 (Figure 4.4A). Few GC nuclei stained positively for TUNEL in control and EGF treated follicles with no difference observed between the treatment groups (Figure 4.4B). There was also no significant difference between the proportions of GCs stained positive for cleaved caspase-3 with EGF.
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4.2.2 Inhibition of EGFR reduces baseline and EGF stimulated growth

EGFR is present at both the transcript and protein level in isolated preantral follicles (see Chapter 3) therefore the implications of inhibiting EGFR activity with the specific inhibitor AG1478 on both baseline and EGF stimulated follicle growth were investigated. AG1478 is a potent cell-permeable inhibitor of EGFR, it functions as a reversible ATP-competitive inhibitor highly selective for EGFR and doesn't cross-react with ErbB2 up to 100 µM. Previous work from the PhD thesis of Victoria Atess (PhD Thesis, 2015) carried out follicle cultures combining EGF with AG1478 treatment, however no separate inhibitor alone control was included in these experiments. Therefore, in light of the work in Chapter 3 revealing the presence of EGF-like ligands within the isolated preantral follicle, these experiments were repeated but with the inclusion of AG1478 added to culture alone without ligand. This had the additional benefit of investigating any detrimental effects of AG1478 on follicle health that may

**Figure 4.4** EGF has no effect on apoptosis markers TUNEL/Cleaved Caspase-3 in preantral follicles in vitro.

A) Follicles cultured in vehicle control or EGF (10 ng/ml) were fixed and stained for TUNEL (green) and cleaved caspase-3 (red), counterstained with the nuclear marker DAPI (blue), representative images at 24 hours and 72 hours are shown, scale bar = 50 µm. B) Proportion of nuclei stained positively for TUNEL or cleaved caspase-3 in control or EGF treated follicles at 24 hours (n = 7 - 10 follicles) and 72 hours (n = 7 follicles). Bars shown are medians, statistically analysed using Mann Whitney test.
contribute to the findings. Figure 4.5 contains two follicle cultures courtesy of Victoria Atess (PhD Thesis, 2015), combined with one more independent follicle culture, which included all four treatment groups. At all time points AG1478 significantly reduced EGF stimulated follicle growth (Figure 4.5). At 24 hours, AG1478 significantly inhibited follicle growth when added to culture alone. A limitation of this analysis is that the AG1478 alone treatment group was only included in one experimental repeat and thus should be repeated further to confirm.

![Figure 4.5](image.png)

**Figure 4.5 EGFR inhibition reduces baseline and EGF stimulated follicle growth.**
Change in follicle area with AG1478 (10 µM) added alone or in combination with EGF (10 ng/ml). Values shown are mean ± SEM. Statistical analysis between treatments were made within each time point by one-way ANOVA and Tukey’s multiple comparison test, where *p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001. Stars above bars represent statistical difference from vehicle control. Numbers in brackets are the number of follicles in each treatment group combined over 3 separate experiments (n = 2 experiments courtesy of Victoria Atess (PhD Thesis, 2015), n = 1 experiment repeated by Kacie Thomson, each experimental repeat contained 4-6 ovaries and approximately 30 follicles / treatment).

Although follicles appeared morphologically healthy, it was important to clarify if the suppression in baseline follicle growth seen with AG1478 at 24 hours was caused by a toxic effect of the inhibitor on follicle growth. To test this, follicles were collected at the completion of culture at 72 hours and immunostained for apoptosis markers TUNEL and cleaved caspase-3 (Figure 4.6A). There was no difference observed in TUNEL or cleaved caspase-3 staining in the cultured preantral follicles with AG1478 (Figure 4.6B).
Figure 4.6 AG1478 has no effect on apoptosis markers TUNEL/Cleaved caspase-3 in preantral follicles in vitro.

A) Follicles cultured in vehicle control or AG1478 (10 µM) were fixed and stained for TUNEL (green) and cleaved caspase-3 (red), counterstained with DAPI (blue), representative images at 72 hours are shown, scale bar = 50 µm. B) Proportion of nuclei stained positively for TUNEL or cells stained positively for cleaved caspase-3 in control or EGF treated follicles. Bars shown are medians (n = 9 - 10 follicles), statistically analysed using Mann Whitney test.

4.2.3 Inhibition of ErbB2 reduces EGF stimulated follicle growth

The ErbB protein screen in Chapter 3 revealed two ErbB subtypes present in the preantral follicle, EGFR and ErbB2. Although the EGFR is the classic receptor known to be bound and activated by the EGF-like ligands, the high expression of ErbB2 protein in the d16 ovary led to the investigation of the importance of EGFR-ErbB2 heterodimers in relaying the EGF response in preantral follicle growth. ErbB2 inhibitor ii is a cell-permeable small molecular inhibitor of ErbB2, targeting the ATP-biding site of the tyrosine kinase domain of ErbB2. ATP-biding sites are highly conserved across the receptor tyrosine kinases, however ErbB2 inhibitor ii was chosen for its specificity, with no cross-reactivity with EGFR as high as 100 µM reported in mammary carcinoma cell lines (Cheng et al., 2007b).

Work conducted as part of the PhD thesis of Victoria Atess examined the effect of ErbB2 inhibitor ii on baseline and EGF stimulated follicle growth and found no change in either with ErbB2 inhibition. The concentrations used by Atess were 0.5, 1, 5 and 10 µM. The
reported phosphorylation IC\textsubscript{50} value, i.e. the concentration at which the inhibitor elicits half maximal effect, for ErbB2 inhibitor ii was 6.6 µM for phosphorylation, but the IC\textsubscript{50} value for cell growth was reported at 30.9 µM in mammary carcinoma cell lines (Cheng et al., 2007b). A dose response was therefore carried out with ErbB2 inhibitor ii at higher concentrations than used previously (20 and 40 µM). Up to 40 µM the ErbB2 inhibitor had no significant effect on baseline follicle growth (Figure 4.7A). When added in combination with EGF (10 ng/ml), inhibition of ErbB2 significantly reduced EGF stimulated follicle growth at 24 hours, but not significantly at 48 or 72 hours (Figure 4.7B).

\textbf{Figure 4.7 ErbB2 inhibition reduces EGF stimulated preantral follicle growth.}  
A) Increasing doses of ErbB2 inhibitor ii (20 and 40 µM) on baseline follicle growth (n = 6 ovaries). B) ErbB2 inhibitor ii (20 and 40 µM) added in combination with EGF (10ng/ml) (n = 10 ovaries). Values shown are mean ± SEM. Statistical analysis between treatments were made within each time point by one-way ANOVA and Tukey’s multiple comparison test, where ** p < 0.01, **** p < 0.0001. Stars above bars represent statistical difference from vehicle control. Numbers in brackets are the number of follicles in each treatment group.
4.2.4 EGF stimulates MAPK signalling in preantral follicles in vitro

An array of signalling cascades can be activated downstream of the ErbBs dependent on the ligand identity, the dimer composition and the phosphotyrosine binding proteins recruited post-binding. The MAPK pathway is common to ErbB signalling, activated downstream of all ErbB dimer combinations. Additionally, the MAPK pathway has been proposed as the most important pathway in mediating the biological response of the EGFR (Wee and Wang, 2017). It was investigated if the MAPK pathway was integral to the stimulation in preantral follicle growth observed with the addition of EGF.

U0126 is a highly selective and potent inhibitor of MEK1/2 (Favata et al., 1998). Initially, a preantral follicle culture examining the effect of increasing concentrations of U0126 on follicle growth was undertaken. There was no significant change in growth with U0126 at 0.1 µM, 1 µM or 10 µM (Figure 4.8A). The concentration of 10 µM was chosen for further experimentation to remain consistent with previous literature published in the mouse ovary (Wang et al., 2013, Fan et al., 2004, Su et al., 2002). However, it should be noted that at 48 and 72 hours there was a trend toward decreased follicle growth with 10 µM U0126 and it would be interesting to repeat this experiment with lower concentrations of the inhibitor. When added in combination with EGF (10 ng/ml), inhibition of MEK1/2 significantly reduced EGF stimulated follicle growth at 24 and 48 hours (Figure 4.8B). The inactive analogue U0124, included as a negative control, had no effect on baseline or EGF stimulated follicle growth.
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**Figure 4.8 MEK1/2 inhibition reduces EGF stimulated follicle growth.**

A) The effect of increasing concentrations of U0126 (0.1, 1 and 10 µM) on preantral follicle growth in vitro (n = 6 ovaries). B) EGF (10 ng/ml) added in combination with either U0126 (10 µM) or its inactive analogue U0124 (10 µM) (n = 6 ovaries). Values shown are mean ± SEM. Statistical analysis between treatments were made within each time point by one-way ANOVA and Tukey’s multiple comparison test, where * p < 0.05 **, p < 0.01, *** p < 0.001, **** p < 0.0001. Stars above bars represent statistical difference from vehicle control. Numbers in brackets are the number of follicles in each treatment group.

The MAPK cascade is fundamental in the downstream response to a wide array of extracellular stimuli. Inhibition of total MAPK activation with U0126 in the preantral follicle culture is likely to not only ablate EGF signalling but also other unspecific upstream stimuli either within the culture media or from factors produced locally within the follicle. Although work described in Figure 4.8 gives strong evidence for EGF stimulation of the MAPK cascade, another approach looking at downstream activation of MAPK through ERK1/2 phosphorylation was undertaken in EGF treated follicles.
Follicles were mechanically isolated from d16 ovaries, pooled into groups of 40 and transferred to low protein binding Eppendorf tubes (see Chapter 2.3.2.2). Grouped follicles were treated with either vehicle control or EGF (10 ng/ml) for 15 minutes then washed in ice cold PBS and protein extracted. Total protein lysate was separated by gel electrophoresis and visualised through western blotting probed for phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated AKT (p-AKT) (Figure 4.9A). There was no significant increase in p-ERK1/2 or p-AKT with EGF treatment (Figure 4.9B). Although visually there appeared to be an increase in p-ERK1/2, this was not consistent in each ovary repeat and was not significant.

**Figure 4.9 EGF effect on ERK1/2 and AKT phosphorylation in preantral follicles.**
A) Response in ERK1/2 and AKT phosphorylation in isolated preantral follicles treated with EGF (10 ng/ml) or vehicle control for 15 minutes shown by western blotting. B) Quantification of ERK1/2 and AKT phosphorylation in follicles following EGF treatment. Signal intensity calculated relative to calnexin as internal loading control, and justified to control sample of d16 ovary lysate ran at identical concentration on each blot (n = 6 ovaries, 40 isolated follicles from same ovary pooled). Bars shown are mean ± SEM, statistical analysed by unpaired t-test.

### 4.2.5 EGF promotes widespread effects on gene expression in cultured preantral follicles

In order to assess whether the increase observed in follicle growth was accompanied by regulation of gene expression, preantral follicles cultured in the presence or absence of EGF were analysed by quantitative RT-PCR. Previous unpublished work from within the lab found that changes in gene expression with treatment were often seen earlier during the culture but lost upon culture completion at 72 hours. Therefore, an
additional follicle culture with EGF was performed with samples collected at 24 hours, pooled into groups from the same mouse and RNA extracted.

Initially, response to EGF was confirmed by growth analysis to ensure the culture conditions replicated what was previously observed (Figure 4.10). Interestingly, Figure 4.10 revealed that although there was consistent elevation in follicle size with EGF, the growth response varied considerably between mice. As mice are obtained from cross-fostered litters this may be due to slight variations in age and follicle composition of the ovary.

![Graph showing follicle area change](image)

**Figure 4.10 EGF stimulates preantral follicle growth at 24 hours.** Percentage change in follicle area relative to 0 hour, data is shown from individual mice. Values are mean ± SEM ($n = 31 - 38$ follicles / mouse), statistical analysis between treatments were made within each time point by unpaired t-test where **** $p < 0.0001$.

The dramatic increase seen in GC proliferation with EGF led to an initial investigation into the expression of genes involved in cell cycle progression. Regulators of the cell cycle, *Ccnd2*, which regulates cyclin-dependent kinases, and *Cdkn1b*, a cell cycle inhibitor, were both significantly decreased in the EGF treated follicles relative to control (Figure 4.11). Levels of mRNA for genes associated with apoptosis were also
investigated, and found pro-apoptosis related genes *Bad* and *Bax* remained unchanged in EGF treated follicles, while the anti-apoptotic gene *Bcl2* was increased.

**Figure 4.11 Effect of EGF on gene expression of regulators of the cell cycle and apoptosis in cultured preantral follicles.**

Preantral follicles cultured with or without EGF (10 ng/ml) for 24 hours. Gene expression data has been log$_2$ transformed and shown as fold change in EGF treated follicles relative to control. Bars represent mean ± SEM (n = 5 ovaries, 40 follicles from same ovary pooled). Statistical analysis between control vs. EGF by unpaired t-test, where ** p < 0.01, *** p < 0.001.

ERBB2 protein first appears at the interface of cuboidalising GCs, with a strikingly similar expression pattern to that observed in N-cadherin (Figure 3.6 and Figure 1.2) (Mora et al., 2012). It was hypothesised that ErbB2 may play a role in regulating the assembly of N-cadherin based adherens junctions. Levels of N-Cadherin (encoded by *Cdhd2*) as well as other genes important in regulating cell adhesion in follicles, including E-cadherin (encoded by *Cdhd1*) and connexin 43 (encoded by *Gja1*) were investigated. In EGF exposed preantral follicles *Cdhd1*, *Cdhd2* and *Gja1* were all significantly decreased relative to control (Figure 4.12).
A screen identifying EGF responsive genes in rat ovarian surface epithelial cells highlighted some potential EGF target genes associated with the cytoskeleton (Abdollahi et al., 2003). Significant elevations in Ezr, encoding Ezrin, a protein that plays a key role in cell surface structure adhesion, migration, and organization, and Tuba1, a major constituent of microtubules, were observed in EGF treated follicles, whereas Tuba3 was significantly decreased (Figure 4.12). Cfl1 encoding Cofilin 1, an intracellular actin-modulating protein, was also unchanged with EGF.

The MMPs and their tissue inhibitors (TIMPs) have been postulated to play a critical role in ECM remodelling during follicular development, and were also highlighted in the rat OSE as EGF regulated (Abdollahi et al., 2003). There was no change in Mmp14 levels, but Timp1 was significantly up-regulated with EGF (Figure 4.12).

Levels of Adam17 mRNA were investigated owing to its importance in the processing of EGFR ligands from their transmembrane precursors, such as TGF-α and HB-EGF (Lee et al., 2003). EGF exposure resulted in decreased Adam17 gene expression (Figure 4.12).
Investigation into the effect of EGF on growth factor receptors revealed widespread down-regulation of Egfr, Erbb2, Amhr2 and Fshr (Figure 4.13). Fshr was included in this screen, as although a gonadotropin receptor stimulation of FSHR has been shown to stimulate preantral follicle growth in vitro (Hardy et al., 2017, Spears et al., 1998, Cortvrindt et al., 1997, Wright et al., 1999).

EGF caused widespread down-regulation of growth factor ligands, decreasing mRNA levels of Tgfb2, Gdf9 and Bmp15 and most strikingly Amh, which was reduced 4-fold in EGF treated follicles. Additionally, EGF exposure resulted in decreased levels of EGF-like ligands Tgfa and Hbegf mRNA, but had no significant effect on Egf, Btc or Ereg. There was one notable exception to the global down-regulation of growth factor ligands,
connective tissue growth factor, *Ctgf*, which was increased 2-fold in EGF treated follicles (Figure 4.13).

![Figure 4.13 Effect of EGF on the expression of growth factor ligand and growth factor receptor genes.](image)

**Figure 4.13 Effect of EGF on the expression of growth factor ligand and growth factor receptor genes.**

Follicles were cultured with or without EGF (10 ng/ml) for 24 hours. Gene expression data has been log₂ transformed and shown as fold change in EGF treated follicles relative to control. Bars represent mean ± SEM (n = 5 ovaries, 40 follicles from same ovary pooled). Statistical analysis between control vs. EGF by unpaired t-test, where * p < 0.05, ** p < 0.01, *** p < 0.001.

EGF has been postulated to play a role in promoting steroidogenesis in GCs (Boland and Gosden, 1994), and has been shown to regulate gonadotropin-induced steroidogenesis in the male and female gonads (Jammongjit et al., 2005). Transcript levels of steroidogenic enzyme *Cyp11a1* were decreased in EGF treated follicles, while *Cyp19* and *Star* remained unchanged (Figure 4.14). Additionally, levels of steroid receptors were investigated revealing that *Ar, Esr1* and *Esr2* were all decreased in EGF exposed follicles.
Figure 4.14 Effect of EGF on the levels of genes encoding steroid receptors and steroidogenic enzymes.

Follicles were cultured with or without EGF (10 ng/ml) for 24 hours. Gene expression data has been log$_2$ transformed and shown as fold change in EGF treated follicles relative to control. Bars represent mean ± SEM (n = 5 ovaries, 40 follicles from same ovary pooled). Statistical analysis between control vs. EGF by unpaired t-test, where * p < 0.05, *** p < 0.001.

Finally, the effect of EGF on transcription factors and downstream signalling pathways was investigated. Snail is a zinc-finger transcription factor that is known to be a crucial player during EMT (Liu et al., 2014). Work from our group has proposed that follicles undergo partial EMT following activation (Mora et al., 2012). Early growth response protein 1 encoded by Egr1 is a transcription factor, known to target genes required for differentiation and mitogenesis, and was up-regulated 11.1-fold in the rat OSE with EGF (Abdollahi et al., 2003). There was no change observed in Snai1 mRNA levels but a 3-fold increase in Egr1 with EGF (Figure 4.15).

Upon ligand binding, an array of signalling pathways are activated downstream of the ErbBs. Some members of the MAPK cascade were highlighted as being regulated by EGF in the rat OSE (Abdollahi et al., 2003). No difference was observed in the transcript
levels of Mapk1, Mapk14 or Map2k2 with EGF in cultured follicles (Figure 4.15). Additionally, there was no change in Calm1, encoding Calmodulin 1, the calcium-modulating protein, or Camk4, encoding the enzyme Calcium/calmodulin-dependent protein kinase type IV (Figure 4.15). Finally mRNA levels of Tob1 were investigated in EGF treated follicles. Tob1 encodes a member of the tob/btg1 family of anti-proliferative proteins that is inactive downstream of ErbB2. No change was observed in Tob1 with EGF (Figure 4.15).

**Figure 4.15 Effect of EGF on genes encoding transcription factors and downstream signalling pathways.**

Follicles were cultured with or without EGF (10 ng/ml) for 24 hours. Gene expression data has been log2 transformed and shown as fold change in EGF treated follicles relative to control. Bars represent mean ± SEM (n = 5 ovaries, 40 follicles from same ovary pooled). Statistical analysis between control vs. EGF by unpaired t-test, where *** p < 0.001.
**4.2.6 EGF alters protein expression in cultured preantral follicles**

The widespread effect of EGF on gene expression in preantral follicles prompted the investigation into variations in protein expression during culture. Follicles were collected at 24 and 72 hours and fixed for immunohistochemical examination.

One of the most pronounced effects detected in mRNA was that of *Amh* that was decreased 4-fold following EGF treatment (Figure 4.13). AMH is one of the characteristic proteins of growing follicles, critical in inhibiting follicle activation, making it an intriguing target of EGF. AMH protein was dramatically reduced at both 24 and 72 hours in EGF treated follicles compared to control (Figure 4.16).

![Figure 4.16 Immunolocalisation and quantification of AMH protein in preantral follicles treated with EGF.](image)

**Figure 4.16 Immunolocalisation and quantification of AMH protein in preantral follicles treated with EGF.** Preantral follicles cultured with EGF (10 ng/ml) or vehicle control for 24 or 72 hours. A) Sections of follicles immunolabelled for AMH (green) and counterstained with DAPI-labelled nuclei (blue). Scale bar = 50 μm. B) Quantification of AMH using ImageJ, calculated as percentage positive staining above a threshold in the GC compartment (n = 5 - 15 follicles). Bars represent mean ± SEM, statistically analysed by unpaired t-test within time points, where ** p < 0.01, *** p < 0.001.

The reduction observed in the transcript levels encoding the ErbBs with EGF, was reflected in ERBB2 protein expression. EGF treatment significantly reduced immunostaining for ERBB2 protein at 24 hours but interestingly not at 72 hours (Figure 4.17). Unfortunately, EGFR protein could not be reliably detected by
immunofluorescence within mouse GCs using commercial antibodies, and further investigation is required.

Figure 4.17 Immunolocalisation and quantification of ERBB2 protein in preantral follicles treated with EGF.

Preantral follicles cultured with EGF (10 ng/ml) or vehicle control for 24 or 72 hours. A) Sections of follicles immunolabelled for ERBB2 (green) and counterstained with DAPI-labelled nuclei (blue). Scale bar = 50 µm. B) Quantification of ERBB2 using ImageJ, calculated as percentage positive staining above a threshold in the GC compartment (n = 9 - 17 follicles). Bars represent mean ± SEM, statistically analysed by unpaired t-test within time points, where *** p < 0.001.

Quantitative RT-PCR demonstrated that Cdh2 encoding adherens junction N-CADHERIN was reduced at 24 hours with EGF (Figure 4.12). However, immunofluorescence staining for N-CADHERIN revealed a significantly increase in protein expression at 72 hours following EGF treatment (Figure 4.18).

The protein expression of CONNEXIN 43, although variable between follicles, was found to be significantly decreased with EGF at 24 hours (Figure 4.19). This finding coincides with the decrease seen in Gja1 transcript levels in EGF treated follicles though quantitative RT-PCR (Figure 4.12).
Figure 4.18 Immunolocalisation and quantification of N-CADHERIN protein in preantral follicles treated with EGF.
Preantral follicles cultured with EGF (10 ng/ml) or vehicle control for 24 or 72 hours. A) Sections of follicles immunolabelled for N-CADHERIN (green) and counterstained with DAPI-labelled nuclei (blue). Scale bar = 50 µm. B) Quantification of N-CADHERIN using ImageJ, calculated as percentage positive staining above a threshold in the GC compartment (n = 6 - 11 follicles). Bars represent mean ± SEM, statistically analysed by unpaired t-test within time points, where * p < 0.05.

Figure 4.19 Immunolocalisation and quantification of CONNEXIN 43 with EGF in preantral follicles in vitro.
Preantral follicles cultured with EGF (10 ng/ml) or vehicle control for 24 or 72 hours. A) Sections of follicles immunolabelled for CONNEXIN 43 (green) and counterstained with DAPI-labelled nuclei (blue). Scale bar = 50 µm. B) Quantification of CONNEXIN 43 using ImageJ, calculated as percentage positive staining above a threshold in the GC compartment (n = 7 - 11 follicles). Bars represent median, statistically analysed by Mann Whitney test, where * p < 0.05.
Finally, protein expression of AR was investigated in EGF treated follicles. In contrast to what was observed at the mRNA level, AR immunostaining was significantly increased at 24 hours with EGF treatment (Figure 4.20).

**Figure 4.20 Immunolocalisation and quantification of AR with EGF in preantral follicles in vitro.**

Preantral follicles cultured with EGF (10 ng/ml) or vehicle control for 24 or 72 hours. A) Sections of follicles immunolabelled for AR (green) and counterstained with DAPI-labelled nuclei (blue). Scale bar = 50 µm. B) Quantification of AR using ImageJ, calculated as percentage positive staining above a threshold in the GC compartment (n = 9 - 10 follicles). Bars represent mean ± SEM, statistically analysed by unpaired t-test within time points, where * p < 0.05.

### 4.3 Discussion

#### 4.3.1 EGF stimulates GC proliferation *in vitro* resulting in follicle growth

The present study has shown that supplementation of culture media with EGF ligand significantly increased mouse preantral follicle growth in culture. This finding is supported by previous work undertaken in livestock species. The growing importance of IVF in modern agricultural practices has driven forward research optimising follicle culture conditions, and supplementation with EGF has been shown to increase follicle size in isolated preantral follicle cultures isolated from cow (Gutierrez et al., 2000), pig (Santos et al., 2014), horse (Aguiar et al., 2017) and goat (Celestino et al., 2011, Silva et al., 2013) ovary.

The increase in preantral follicle size observed with EGF was a consequence of increased GC proliferation. Oocytes typically reach maximal size around the formation
of the antral cavity (Picton et al., 2008), however oocyte growth was minimal over the culture period and the increase observed in follicle size was attributed to a rise in GC multi-layering. In support of this, the proportion of GCs stained positively for proliferation marker Ki67 increased in follicles exposed to EGF. The decrease seen with EGF in Cdkn1b gene expression, a cell cycle inhibitor (p27Kip1) that binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, may provide a mechanism by which EGF encourages cell cycle progression at G1. Unexpectedly, there was a coinciding decrease in Ccnd2 mRNA levels encoding Cyclin D2 protein. Cyclin D2 is known to play an essential role in gonadal cell proliferation as Cyclin D2 knock out female mice are sterile owing to the inability of ovarian GCs to proliferate (Sicinski et al., 1996). The decrease in Ccnd2 is perplexing, however it is likely that the activity and degradation of these cell cycle proteins is equally if not more important than their transcription. Evidence from epithelial cancer cells has shown that overexpression of EGFR activates p27 proteolysis and Ras-driven p27 proteolysis, down-regulating Cdkn1b post-transcriptionally, and promoting subsequent cellular proliferation (Chu et al., 2008). Expression and activity of cell cycle proteins could be examined in preantral follicles to gain a clear picture of the mechanisms driving GC proliferation. The transcription factor Egr1 was also increased in EGF stimulated follicles. EGR-1 is known to target genes required for differentiation and mitogenesis and has been shown previously as EGF responsive, up-regulated 11.1-fold in the rat OSE (Abdollahi et al., 2003), providing another mechanism underlying EGF stimulated cell division. The EGF-induced elevation in GC proliferation was in agreement with previous publications, reporting increased DNA quantification in cultured porcine GCs (Morbeck et al., 1993), increased PCNA stained nuclei in porcine preantral follicle cultures (Mao et al., 2004) and increased thymidine incorporation by GCs of bovine preantral follicles (Wandji et al., 1996a).

Follicle viability assessed through dual staining for TUNEL and cleaved caspase-3 protein revealed no difference between treatments, however a significant increase in Bcl2 mRNA was reported with EGF. Bcl-2 is considered an anti-apoptotic protein, and has been associated with the progression of numerous cancers. Furthermore, EGF in the literature has been shown to increase follicle viability in porcine (Mao et al., 2004) ovine (Andrade et al., 2005, Santos et al., 2014), equine (Aguiar et al., 2017), caprine
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(Celestino et al., 2009), feline (Fujihara et al., 2014) and murine (Tilly et al., 1992) ovarian tissue. Amplified Bcl2 transcription may encourage a subtle increase in GC viability and contribute to the expansion observed in the GC compartment. However, the lack of change in protein apoptosis markers indicates that the increase in follicle size with EGF is driven by increased GC proliferation rather than reduced apoptosis.

4.3.2 EGF signals through EGFR homodimers and EGFR-ErbB2 heterodimers

EGFR is known to be the principal receptor for the EGF-like ligands (Yarden and Sliwkowski, 2001), and indeed the EGFR inhibitor AG1478 ablated EGF stimulated follicle growth at all time points. The addition of an ErbB2 inhibitor reduced EGF stimulated follicle growth at 24 hours during the most pronounced period of GC proliferation, suggesting that EGFR-ErbB2 heterodimers are involved in relaying the EGF signal. Although ErbB2 homodimers are possible this process is ligand-independent, as ErbB2 lacks a known ligand, and likely isn’t involved in EGF induced signalling. Recent structural studies suggest that the structure of ErbB2 resembles a ligand-activated state making ErbB2 the preferred dimerisation partner of all the ErbBs. Additionally, when ErbB2 is highly expressed ErbB heterodimers can form preferentially over homodimers (GrausPorta et al., 1997). The co-expression of EGFR and ErbB2 protein in the pre-pubertal mouse ovary (see Chapter 3) makes heterodimerisation upon EGF stimulation highly possible. This novel result is the first to implicate ErbB2 in relaying the EGF signal in preantral follicles. The relative importance of homo- and hetero- dimers in EGF stimulated follicle growth may be elucidated in future studies using an antibody that blocks ErbB2 dimerisation such as Pertuzumab, hence inhibiting EGFR-ErbB2 heterodimerisation. Pertuzumab binds at the dimerisation arm of ErbB2 and is typically administered in the treatment of HER-2 positive breast cancer, but may be added in combination with EGF to preantral follicle cultures.

Following EGF exposure, mRNA and protein expression of the ErbBs was decreased suggesting a negative feedback mechanism within the follicle, de-sensitising itself to EGF signalling and over-growth. In goat preantral follicles EGF treatment causes similar reductions in Egfr mRNA (Silva et al., 2013). At steady-state cell growth in other tissue types, ErbBs are constitutively internalized and recycled back to the cell membrane at a comparable rate to the level of basal membrane recycling (Sorkin and Goh, 2009).
However, upon EGF stimulation, EGFR is rapidly ubiquitinated and internalised from the cell membrane (Wiley et al., 1991). The EGFR is then targeted toward lysosomal degradation, resulting in global down-regulation at the cell surface (Ebner and Derynck, 1991). Hence EGF-stimulated degradation of EGFR-ERBB2 heterodimers may explain the drop seen in ERBB2 protein with EGF. However, EGFR heterodimers containing ErbB2 have been described previously as endocytosis impaired as they preferentially evade lysosomal degradation, and instead are recycled back to the cell membrane (Waterman et al., 1998). The decrease in ErbB2 protein may therefore be linked to repression in Erbb2 transcription. Interestingly, the proportion of Ki67 positive nuclei with EGF was significantly increased only at 24 hours, corresponding to the initial sharp increase recorded in follicle size. At 72 hours Ki67 staining was not significantly elevated, coinciding with the plateau seen in EGF stimulated growth at 48 and 72 hours. This plateau may be explained by the down-regulation of ErbB receptors. Future studies should examine the effect of EGF treatment of EGFR protein in mouse preantral follicles.

Another potential mechanism involves modulation of upstream ErbB regulators such as oocyte-specific Gdf9 and Bmp15, the mRNA of which is decreased with EGF supplementation. GDF9 and BMP15 have been shown to stimulate EGFR expression in mouse cumulus cells (Su et al., 2010) hence the reduction in Gdf9 and Bmp15 with EGF may result in the subsequent decrease in EGFR by indirect regulatory mechanisms.

To investigate the presence of endogenous EGF signalling within the preantral follicle, ErbB inhibitors were added to culture without ligand stimulation. The EGFR inhibitor AG1478 significantly reduced baseline follicle growth, suggesting endogenous activation of the EGFR. There was no difference observed in TUNEL or cleaved caspase 3 staining in cultured follicles with AG1478, suggesting the smaller size was caused by a reduction in stimulation of GC proliferation. The most highly expressed EGF-like ligands detected within the preantral follicle were Tgf-α, Hb-egf, Egf and Btc (see Chapter 3), which may be active during culture moderating growth. Sasseville et al., found reduced \[^{3}H\]thymidine incorporation of preantral secondary follicles in the presence AG1478 (5 μM) (Sasseville et al., 2010). They reported that pharmacological inhibition of EGFR with AG1478 reduced oocyte stimulated mitogenic action of GCs. Specifically, they identified members of the TGFβ family (GDF9, Activin A and TGFβ1) which stimulated \[^{3}H\]thymidine intake in GCs from mural antral follicles and whose effects were reduced.
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with AG1478. Therefore, the depression in baseline follicle growth seen in the present study may be through the inhibition of both EGF-like and TGFβ ligand stimulation. The ErbB2 inhibitor had no effect when added to culture alone, indicating that ErbB2 is either not involved in EGF-like and TGFβ endogenous signalling, or that its loss can be compensated for by EGFR homodimers. However, ERBB2 protein is highly expressed in the d16 ovary and spatially and temporally regulated, localised to growing preantral follicles and it cannot be excluded that ErbB2 may play additional roles in the regulation of preantral follicle development, such as modulating cell adhesion, steroidogenesis or the partial EMT following activation. Further investigation into the role of ErbB2 should examine downstream changes in morphology and protein expression in cultured preantral follicles with ErbB2 inhibitors.

Inhibition of the MAPK signalling cascade with the MEK1/2 specific inhibitor U0126 ablated EGF stimulated follicle growth. The MAPK/ERK cascade is directly activated downstream of all ErbB dimer combinations (Schulze et al., 2005), so MEK1/2 inhibition likely reduces signalling from both EGFR homodimers as well as EGFR-ErbB2 heterodimers. The ligand-activated MAPK cascade culminates in the phosphorylation of ERK1/2. ERK1/2 interacts with a wide variety of substrates including transcription factors to initiate responses in growth, proliferation, differentiation, migration, and inhibition of apoptosis. It has been proposed that the most important pathway mediating the EGFR biological response is the MAPK cascade (Wee and Wang, 2017), which agrees with the ablation of EGF-stimulated follicle growth seen with U0126. Controlled activation of the RAS/MAPK pathway in GCs is essential for female fertility (Fan et al., 2009). GC specific knock-ins of RAS protein in small growing follicles revealed that constitutively active Ras causes arrested follicular growth at the early stages of development resulting in infertility in mice by 3 months of age (Fan et al., 2008). EGF may therefore play an important role in regulating MAPK signalling in preantral follicles. Some members of the MAPK cascade were highlighted as EGF regulated in the rat OSE (Abdollahi et al., 2003), Mapk1, Mapk14 and Map2k2 were up-regulated 3.1 fold, 2.8-fold and 3.3-fold respectively. The current study detected no change in the transcript levels of Mapk1, Mapk14 or Map2k2 in cultured preantral follicles. However, it is possible that the phosphorylation states of these proteins is more biologically relevant than their overall expression.
As mentioned previously, inhibition of the MAPK cascade in vitro may elicit unspecific effects, inhibiting basal response to extracellular stimuli within the culture media or produced locally within the follicle. Activation of ERK1/2 and thus the MAPK cascade in preantral follicles was investigated with short EGF treatment, however no significant increase was observed in ERK1/2 phosphorylation. The large variability in ERK1/2 phosphorylation observed between ovaries is likely caused by a technical constraint, owing to the difficulty in collecting follicles following EGF exposure. After treatment, follicles were spun down and ice cold PBS was added to halt cellular activity, however some replicates required longer centrifugation if follicles became adhered to the side of the Eppendorf, meaning the phosphorylation levels were detected at longer time points in some biological repeats. Later studies in Chapter 6 optimised and put to use a culture system for GCs isolated from antral follicles. These GCs responded rapidly to EGF treatment (used as positive control), with elevations in ERK1/2 phosphorylation seen 2 minutes after exposure (Figure 8.10).

It should be noted that EGFR and ErbB2 are also known to signal through the p70S6K/p85S6K pathway and indirectly stimulate the PI3K-activated AKT pathway to mediate their response (Yarden and Sliwkowski, 2001). Inhibitors of these pathways in combination with EGF could be the focus of further investigation. However, there was no change in AKT phosphorylation following EGF treatment in either preantral follicles or GCs from antral follicles, indicating that the PI3K-activated AKT pathway is not the principal pathway relaying the response to EGF. Furthermore, previous studies have shown that EGF signals principally through the MAPK pathway to stimulate proliferation in feline ovarian tissue in stromal cells (Fujihara et al., 2014). Taken together, these results suggest that EGF signals through both EGFR homodimers and EGFR-ErbB2 heterodimers to stimulate the MAPK cascade and induce a mitogenic response in the GCs of mouse preantral follicles.

### 4.3.3 EGF network in cell adhesion and epithelial-mesenchymal transition following activation

The strikingly similar protein localisation pattern of ERBB2 and N-CADHERIN (Mora et al., 2012) led to the hypothesis that the EGF network and ErbB2 may play a role in regulating the assembly of N-cadherin based adherens junctions. Work performed by
Victoria Atess (PhD Thesis, 2015) demonstrated that ErbB2 protein appears first at the interface of cuboidalising GCs (Figure 3.6). N-cadherin protein becomes distinct at this transitional stage, expressed between the elongating membranes of the GCs (Figure 1.2) and is thought to play a crucial role in cuboidalisation (Mora et al., 2012). Mora et al., proposed that a partial EMT takes place following follicle activation as seen by the lack of classic epithelial markers such as E-cadherin, and the coinciding presence of mesenchymal markers such as vimentin and N-cadherin, as well as changes in GC morphology, with looser GC attachments with progressive follicle development. In human prostate and lung cancer cells EGF can induce EMT (Liu et al., 2014). These reports show after treatment with EGF (20 ng/ml) for 24 hours, both PC-3 and A549 cells increased mRNA and protein expression of N-cadherin and fibronectin, with a simultaneous decrease of E-cadherin as both mRNA and protein. The current results show that EGF increases N-CADHERIN protein expression in mouse GCs in cultured preantral follicles, and decreases Cdh1 mRNA levels, encoding E-Cadherin, suggesting that EGF mediates EMT in preantral follicles. In contrast, the results also showed a decrease in Cdh2 mRNA levels with EGF, which suggests that EGF increases N-CADHERIN protein expression through post-transcriptional regulation.

The transcription factor Snail is critical during EMT. In human prostate and lung cancer cells, levels of Snail rapidly increased with EGF exposure, and knock down of Snail reversed these effects indicating that Snail is essential for EGF-induced EMT (Liu et al., 2014). Interestingly, there was no change in Snai1 mRNA with EGF in mouse preantral follicles. Expression and nuclear localisation of SNAIL protein following EGF treatment could be examined, as activated Snail strongly represses the transcription of E-Cadherin (Peinado et al., 2004). Ezrin encoded by Ezr also plays an important role in EGF induced EMT in cancer metastasis (Wang et al., 2014) and was increased 2.8 fold by EGF in the rat OSE (Abdollahi et al., 2003). Elevated Ezr transcription was detected in EGF treated preantral follicles, possibly encouraging EMT. These results taken together indicate that ErbB signalling may have a role to play in mediating EMT following follicle activation.

Connexin 43 (Cx43) is a gap junction protein encoded by the Gja1 gene. Cx43 is expressed in differentiated GCs playing a critical role in the adhesion between germ cells and surrounding somatic cells (Teilmann, 2005), and disruption in the Cx43 gene
disrupts progression of follicles beyond primary stages. EGF has been shown to stimulate Cx43 protein expression during early porcine follicular development (Bolamba et al., 2002) as well as in GCs from preantral rabbit follicles (Kennedy et al., 2003), presumably supporting adhesion in the developing follicle. The current study demonstrates paradoxical results in the mouse, as EGF treatment significantly reduced Gja1 mRNA and Connexin 43 protein in preantral follicles. Cx43 has been proposed as a tumour suppressor gene, found to negatively regulate growth of colon cancer cells (Sirnes et al., 2012). The down-regulation of Cx43 may provide another mechanism by which EGF promotes follicle growth.

4.3.4 EGF stimulates widespread down-regulation of growth factor ligands and receptors

Widespread reductions in growth factor ligands, including those in the TGFβ family Amh, Tgfb2, Tgfb3, Gdf9, Bmp15 and EGF-like ligands Tgfa and Hbegf were observed in EGF treated preantral follicles. The most pronounced reduction was that of AMH with dramatic decreases observed in both mRNA and protein expression. Co-culture of isolated oocytes and GCs demonstrate that AMH is regulated by signals from the oocyte, and specifically AMH expression is stimulated by oocyte-specific GDF9 (Salmon et al., 2004). Furthermore, unpublished work from the lab has shown that removal of the oocyte from preantral follicles dramatically reduces AMH mRNA (Mora and Fenwick, unpublished observations). EGF induced reductions in Gdf9 and Bmp15 mRNA levels may therefore indirectly reduce AMH expression. Additionally, Egf and Tgfa were detected in isolated mouse oocytes and may directly negatively regulate AMH production in GCs. AMH is produced by the GCs of growing follicles (Durlinger et al., 2002b) and acts as an inhibitor of primordial follicle activation (Durlinger et al., 2002a). In mice, in vivo deletion of Amh results in accelerated rates of primordial follicle recruitment and premature depletion of the ovarian reserve (Durlinger et al., 1999). EGF induced AMH reduction may therefore have confounding effects on primordial follicle activation. This hypothesis is explored further in Chapter 5, which examines the effect of EGF treatment on neonatal mouse ovaries.

The global down-regulation of growth factor transcription may be a negative feedback mechanism caused by overstimulation within the follicle to prevent overgrowth.
Interestingly, there was one exception detected as EGF treatment increased mRNA levels of \textit{Ctgf}. Conditional knockout mice have revealed that \textit{Ctgf} expression in the ovary is critical for normal follicle development and deletion of ovarian \textit{Ctgf} results in subfertility (Nagashima et al., 2011). The intraovarian function of \textit{Ctgf} is still unclear, but it is proposed to promote cell growth and angiogenesis (Wandji et al., 2000), and may augment the growth response to EGF. In cultured rat GCs \textit{Ctgf} mRNA is up-regulated by the TGFβ ligands activin A, GDF9 and TGFβ1, however the effect of EGF was not examined. The study also proposed that \textit{Ctgf} played a role in tissue reorganization and ECM deposition during follicular development, (Harlow et al., 2002) which hasn’t yet been examined with EGF but could direct further study.

The availability of growth factor ligands is altered not only by their transcription but also the proteolytic cleavage from their integral membrane precursors. \textit{Adam17} is a metalloprotease enzyme important in the cleavage of soluble ErbB ligands such as TGF-α and HB-EGF from their transmembrane precursors (Lee et al., 2003). mRNA levels for \textit{Adam17} were significantly decreased in EGF treated follicles, which may further diminish availability of the EGF-like ligands. The MMP family are also involved in EGF-like ligand shedding, and MMP14 has been implicated in the membrane release of HB-EGF (Overland and Insel, 2015). There was no change observed in \textit{Mmp14} mRNA, however \textit{Timp1} the metallopeptidase inhibitor was elevated, which may dampen the effects of MMPs mediating EGF-like ligand release, further decreasing availability of growth factor ligands.

Finally, EGF treatment resulted in diminished \textit{Fshr} mRNA levels. FSH signalling is critical for antral follicle development but also plays a physiological role in growth and function of preantral follicles (Hardy et al., 2017). Decreased \textit{Fshr} mRNA expression was also found in the caprine preantral follicle cultured for 6 days with EGF (Celestino et al., 2011), however the literature is conflicting with some reports of increased FSHR mRNA following 18 days of EGF treatment in goat secondary follicles (Silva et al., 2013). These differing results may be explained by the variation in culture duration and follicle stage. In the hamster, EGF has been shown to mediate the mitogenic actions of FSH (Roy and Greenwald, 1991). The current results suggest the action of FSH at the preantral stage of
follicle development is influenced by the presence of EGF, and EGF may render the preantral follicle less sensitive to FSH stimulation.

It is likely that a fine balance exists between stimulatory and inhibitory factors regulating preantral follicle growth and the global down-regulation of stimulatory factors seen with exogenous EGF may be through a primary regulatory mechanism within the follicle to regulate growth.

4.3.5 EGF network in steroidogenesis

Ovarian GCs are the primary source of oestradiol production in the body. EGF has been postulated to play a role in promoting steroidogenesis in GCs, as well as regulating gonadotropin-induced steroidogenesis in the male and female gonads. In oocyte-GC complexes isolated from preantral follicles from d12 mice, EGF increased progesterone, testosterone, and oestradiol levels by 6-fold, 6-fold and 2-fold respectively (Jamnongjit et al., 2005). However the literature is conflicting with some reports stating that EGF can either stimulate oestradiol secretion from GCs (Aguiar et al., 2017), while others report EGF inhibits oestradiol production (Boland and Gosden, 1994, Behl and Pandey, 2001, Silva et al., 2013). In cultured mouse preantral follicles there was a significant decrease in Cyp11a1 mRNA. Cyp11a1 mediates the first reaction in the steroidogenic pathway catalysing the conversion of cholesterol to pregnenolone, considered the rate limiting step, and is expressed primarily in the theca cells. The detected Cyp11a1 mRNA in mouse preantral follicles is likely from attached theca cells during follicle isolation. The down-regulation of Cyp11a1 with EGF may suggest an overall EGF-induced reduction in steroidogenesis. There was no difference observed in Cyp19 mRNA levels in preantral follicles cultured with EGF. Cyp19 encodes the enzyme aromatase, responsible for the aromatisation of androgens to oestrogens in GCs. In agreement, a study from 1988 found that EGF treatment of human GCs obtained from women undergoing IVF, showed no increase in CYP19 mRNA, however EGF inhibited the ability of FSH to stimulate aromatase expression and activity. The decrease seen by others in oestradiol secretion with EGF may be mediated though a global decrease in steroid production caused by lower Cyp11a1 expression, or a reduction of FSH induced aromatisation rather than a direct effect on Cyp19 expression.
EGF treatment significantly decreased expression of steroid receptors, both the oestrogen receptor alpha (Esr1) and beta (Esr2) in preantral follicles. These receptors are critical during GC differentiation and dominance selection in later stage follicles (Tonetta et al., 1985, Couse et al., 2005). Gonadotropins control the expression of ERα and ERβ in the mouse ovary in vivo, and administration of hCG significantly decreased the expression of ERα and ERβ protein levels to induce oocyte maturation (Liu et al., 2017). Interactions between EGF and gonadotropin signalling may indirectly regulate ER expression. Additionally, EGF treatment resulted in decreased Ar mRNA but in contrast increased AR protein expression, suggesting post-transcriptional regulation of AR. Androgen signalling is known to play a stimulatory role during preantral follicle development (see Chapter 6), therefore the increase in AR sensitivity may provide another mechanism by which EGF may stimulate preantral follicle development. Cross-talk between androgens and the EGF family is examined further in Chapter 7.

4.4 Conclusions

In conclusion, mouse preantral follicles in culture are responsive to exogenous EGF. EGF supplementation of culture medium stimulates preantral follicle growth through elevations in GC proliferation. Inhibitor studies showed that both EGFR homodimers and EGFR-ErbB2 heterodimers are responsible in relaying the EGF signal in preantral follicles to activate the MAPK cascade. EGF invokes widespread alterations in gene and protein expression key to follicular development including those associated with cell adhesion, proliferation and steroidogenesis. Expression of EGF-like ligands and ErbB receptor were decreased with EGF treatment, indicating negative feedback loops present within the follicle to prevent overgrowth.
Chapter 5 Effect of EGF on primordial follicle activation

5.1 Introduction
The rate at which primordial follicles are recruited into the growing pool is central in determining the reproductive lifespan of a female. The factors responsible for the maintenance of the quiescent primordial follicle pool and the following steady initiation of growth remain elusive. Inter-follicular signalling is likely to play a crucial role in regulating activation, creating a delicate balance of local inhibitory and stimulatory factors secreted by both growing and primordial follicles.

Detection of ErbB protein in the neonatal d4 ovary (see Chapter 3), populated primarily by dormant primordial follicles prompted the investigation into the role of ErbBs during activation. Additionally, the striking effect of EGF treatment on AMH, a well characterised inhibitor of primordial follicle activation, in cultured preantral follicles (see Chapter 4) led to the hypothesis that the EGF network may have an indirect regulatory effect on follicle activation.

A whole neonatal ovary organ culture system was used as the principal culture method to investigate activation. Cultures were maintained for up to 6 days, at which stage multiple follicles had initiated growth in the medullary region of the ovary. Ovaries were collected and processed for immunohistochemical examination of morphology, activation rate and changes in protein expression. Primordial follicle activation rate was investigated using immunofluorescence detection of the germ cell marker vasa (DDX4) protein and follicle classification based on oocyte size. Work undertaken by Elizabeth Oliver (PhD thesis, 2017) examined the validity of using oocyte area as a surrogate marker for primordial follicle activation in the neonatal ovary culture. A simultaneous analysis using both morphological classification (based on GC number, morphology and degree of multi-layering) and oocyte area revealed that a cut off of 275 µm², whereby follicles with an oocyte area larger than 275 µm² were classified as growing, and follicles with an oocyte area smaller than 275 µm² were classed as primordial, was an appropriate threshold yielding comparable results to morphological follicle
classification. Additionally, using oocyte area for classification proved to be less subjective than morphological analysis as well as more time efficient. Oocyte analysis was performed on two central sections from each ovary and averaged to increase accuracy. To avoid double counting of follicles, sections were selected at least $25 \, \mu m$ apart and only oocytes with a clear nucleus were included.

### 5.1.1 Specific aims

- Investigate the effect of EGF exposure on the rate of primordial follicle activation in the neonatal ovary culture.
- Investigate the effect of inhibiting EGFR with and without EGF on primordial follicle activation during ovary culture.
- Examine the effect of EGF and EGFR inhibition on AMH protein expression in the neonatal ovary.

### 5.2 Results

#### 5.2.1 EGF signalling has no effect on the rate of primordial follicle activation in the cultured neonatal ovary

In order to investigate the role of the EGF network on primordial follicle activation, neonatal mouse ovaries were dissected from d4 mice and cultured in the presence of EGF with or without the EGFR specific inhibitor AG1478. Cultured ovaries were collected after 6 days, fixed, sectioned and stained for the germ cell marker vasa to examine ovarian morphology. Visual inspection of the sections at low and high power suggested that there was no apparent difference in ovarian morphology with EGF, AG1478 or a combination of the two compared to controls (Figure 5.1). Ovaries appeared healthy and remained intact following culture with very few pyknotic nuclei. The ovaries spread and flattened onto the membrane during the culture, although remained circular and were orientated in wax so that multiple central sections could be cut parallel to the disc, rather than perpendicular. Ovaries cultured for 6 days were populated with approximately half primordial follicles and half small growing follicles at the transitional or primary stages of development.
Figure 5.1 Morphology of cultured neonatal ovaries with EGF and/or AG1478. Cultured ovaries were immunolabelled for oocyte-specific vasa protein (DDX4, red) and counterstained with DAPI (blue). Representative images are shown for A) vehicle control, B) EGF (10 ng/ml), C) combined EGF (10 ng/ml) and AG1478 (10 µM) and D) AG1478 (10 µM) alone. E) Magnified area from white box of control ovary showing follicle composition of ovary. F) Magnified image on one growing and two primordial follicles. Scale bar = 100 µm (A-E), 30 µm (F). Arrows indicate primordial follicles and arrowheads indicate growing follicles.

To quantify the rate of primordial follicle activation, the areas of oocytes with clear nuclei were analysed from two central ovarian sections. Overall distribution of oocyte area did not change with treatment (Figure 5.2A). The primordial follicle pool was represented by a peak in follicles with an oocyte area of between 150 and 275 µm². The growing pool is represented by the tail to the right showing oocyte growth following activation, the majority of oocytes remained under 500 µm² with few reaching over 1000 µm². Follicles were classified as either primordial or growing based on the oocyte area cut off of 275 µm², established by Elizabeth Oliver (PhD thesis, 2017).
no significant difference in the proportion of growing follicles with EGF or AG1478 or a combination of the two (Figure 5.2B).

Figure 5.2 Stimulation or inhibition of EGFR does not affect primordial follicle activation in vitro. Neonatal ovaries were cultured for 6 days in vehicle control, 10 ng/ml EGF, combined 10ng/ml EGF and 10 µM AG1478 and 10 µM AG1478 alone. From each cultured ovary two central sections, at least 25 µm apart, were immunolabelled for oocyte-specific vasa protein (DDX4) and area of oocytes with a visible nucleus were measured. A) Distribution of oocyte area using all measurements. Data shows % of oocytes within 100 µm² area bins. Oocyte area was used as a surrogate marker for follicle activation, with a cut off of < 275 µm² classified as primordial, and > 275 µm² classified as growing. B) Data shown is % of growing follicles, averaged first within ovary. Bars are mean ± SEM (n = 3 - 4 ovaries, 6 - 8 analysed sections). Statistically analysed by one-way ANOVA.

Follicle activation is typically accompanied by increased GC cuboidalisation and proliferation. The mitotic activity of GCs in cultured neonatal ovaries was investigated using immunofluorescence detection of proliferation marker Ki67. There was no visual difference in amount of Ki67 staining per follicle with EGF or AG1478 alone or in combination (Figure 5.3), coinciding with the lack of change observed in follicle activation. However, quantitatively analysis of Ki67 in individual follicles could be investigated in future.
Figure 5.3 Stimulating or inhibiting EGF signalling has no visual effect on Ki67 staining in cultured neonatal ovaries.

Neonatal ovaries cultured for 6 days in vehicle control, EGF (10 ng/ml), combined EGF (10 ng/ml) and AG1478 (10 µM) and AG1478 (10 µM) alone were collected and one central section from each ovary was immunolabelled for proliferation marker Ki67 (green) and counterstained with DAPI (blue). Representative images are shown of whole ovary and magnified areas. Scale bar = 100 µm.

5.2.2 EGF decreases average oocyte size in cultured neonatal mouse ovaries

Stimulation with EGF and inhibition of the EGFR with AG1478 had no significant effect on the percentage of follicles that activated over the culture. However, when total oocyte area data was pooled and plotted, EGF had an inhibitory effect on average oocyte area (Figure 5.4). This observation was independent of the presence of AG1478, which had no significant effect on average oocyte area alone when compared to control, and did not ablate the effect of EGF.
Chapter 5: Effect of EGF on primordial follicle activation

Figure 5.4 EGF exposure causes a decrease in average oocyte area in cultured d4 ovaries.

Cultured neonatal mouse ovaries were treated with vehicle control (4 ovaries, 8 sections, n = 670 oocytes), 10 ng/ml EGF (3 ovaries, 6 sections, n = 481 oocytes), combined 10 ng/ml EGF and 10 µM AG1478 (4 ovaries, 8 sections, n = 884 oocytes) and 10 µM AG1478 alone (4 ovaries, 8 sections, n = 527 oocytes) for 6 days. Two central sections, at least 25 µm apart, from each ovary were immunolabelled for vasa (DDX4) protein and area of oocytes with a visible nucleus were measured. Data presented is all oocyte area data points collected and median of treatment groups (horizontal bar). Statistically analysed using Mann-Whitney test, where ** p < 0.01, *** p < 0.001.

5.2.3 EGF signalling does not affect follicle atresia

The decrease observed in average oocyte area with EGF initiated an investigation into the effect of the EGF network on follicle atresia. Immunofluorescence staining for apoptosis marker cleaved caspase-3 was used to assess change in follicle survival with treatment. Qualitatively there was no difference in staining for cleaved caspase-3 in EGF or AG1478 treated ovaries compared to control (Figure 5.5). Cleaved caspase-3 staining was quantified in whole ovaries using ImageJ. The area of positive staining above an established threshold was calculated as a percentage from total area of the ovary.
Cleaved caspase-3 positive staining was very low in all ovaries examined with no significant difference between treatments.

**Figure 5.5 EGF signalling has no effect on apoptosis marker cleaved caspase-3 in cultured neonatal ovaries.**

Neonatal ovaries cultured for 6 days in vehicle control, 10 ng/ml EGF, combined 10 ng/ml EGF and 10 µM AG1478 and 10 µM AG1478 alone. A) One central section from each ovary was immunolabelled for apoptosis marker cleaved caspase-3 (red) and counterstained with DAPI (blue). Representative images are shown. Scale bar = 200 µm. B) Zoomed in image from control section, arrowheads indicate positively stained cells. C) Cleaved caspase-3 staining above a threshold was measured as pixels and calculated as a percentage of total number of pixels from ovarian section. Data shown is individual data point and median (n = 2 - 4 ovaries, 2 - 4 analysed sections).

To further clarify if the reduction in average oocyte area observed with EGF was linked to atresia, total follicle number in each section was recorded. Although fewer sections were analysed in the EGF treatment group, there was no significant difference in the average number of follicles within each section analysed (Figure 5.6).
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Figure 5.6 EGF or EGFR inhibition has no effect on average follicle count per section in cultured neonatal ovary.

Cultured ovaries were treated with vehicle control (4 ovaries, n = 8 sections), 10 ng/ml EGF (3 ovaries, n = 6 sections), combined 10 ng/ml EGF and 10 µM AG1478 (4 ovaries, n = 8 sections) and 10 µM AG1478 alone (4 ovaries, n = 7 sections) for 6 days. Two central sections from each ovary were immunolabelled for vasa (DDX4) protein were used for analysis. Numbers of follicles per section were quantified. Data presented is individual data points and median.

5.2.4 EGF signalling does not visually alter AMH protein expression in cultured neonatal ovaries

The dramatic decrease observed in Amh mRNA and AMH protein expression in cultured preantral follicles with EGF treatment led to the investigation of AMH protein in the cultured neonatal ovary. AMH protein expression is restricted to growing follicles located primarily in the central medulla region of the ovary so one central section was used for examination. Sections of cultured ovary treated with EGF at 10 ng/ml and 100 ng/ml were generously donated by Victoria Atess. There was no difference observed visually in the intensity or localisation of AMH staining with either concentration of EGF (Figure 5.7). Additionally, sections from ovaries cultured with AG1478 with or without EGF ligand were immunostained for AMH protein. Again, visual inspection of the sections suggested that there was no apparent no difference observed in AMH protein expression with AG1478 (Figure 5.8).
Figure 5.7 EGF has no visual effect on AMH protein expression in cultured neonatal ovaries.
Neonatal ovaries cultured for 5 days in vehicle control or EGF (10 ng/ml or 100 ng/ml). Ovaries were collected and one central section from each was immunolabelled for AMH protein (green) and counterstained with DAPI (blue). Representative images are shown (n = 3 ovaries). Scale bar = 200 µm.

Figure 5.8 AG1478 has no visual effect on AMH protein expression in cultured neonatal ovaries.
Neonatal ovaries cultured for 6 days in vehicle control or AG1478 (10 µM) or EGF (10 ng/ml) with AG1478 (10 µM). Ovaries were collected and one central section from each was immunolabelled for AMH protein (green) and counterstained with DAPI (blue). Representative images are shown (n = 2 - 3 ovaries). Scale bar = 200 µm.

5.3 Discussion
5.3.1 EGF signalling has no effect on follicle activation in vitro
Neonatal ovaries cultured in the presence of EGF showed no increase in the proportion of growing follicles, indicating that EGF signalling does not influence mouse follicle
activation. This result is consistent with work reported in the neonatal rat ovary which found that 8 days with 100 ng/mL EGF had no effect on primordial to primary follicle transition (Kezele et al., 2002). Additionally, in feline (Fujihara et al., 2014) and caprine (Silva et al., 2004) ovarian cortical slices, EGF up to 100 ng/ml for 14 and 5 days respectively had no effect on the rate of primordial follicle activation. One study has been published in contradiction of these findings, in the rat neonatal ovary which found ovaries cultured for 8 days with 50 ng/ml EGF showed increase initiation of primordial follicle growth (Li-Ping et al., 2010). Interestingly, the authors reported that siRNA toward c-erbB2 blocked spontaneous and EGF-induced activation of primordial follicles. The current study did not find any effect of inhibition EGFR on follicle activation, however did not investigate inhibition of the ErbB subtype ErbB2.

The d4 mouse ovary expressed ErbB2 protein, and displayed low expression of EGFR (see Chapter 3). The lack of EGFR protein in the d4 ovary may explain the lack of effect seen with EGF and AG1478 treatment. EGF is known to signal principally through the EGFR (Yarden and Sliwkowski, 2001), which was detected only very weakly by western blotting. Furthermore, inhibition of the weak expression of EGFR with AG1478 unsurprisingly had little effect on the cultured neonatal ovary. ErbB2 protein was expressed in the d4 ovary, however its expression did not appear to relay the EGF signal. ErbB2 is unlikely to homodimerise in response to EGF, as it has no known ligands (Klapper et al., 1999) and functions largely as a co-receptor for other ErbBs, which were only weakly detected. Additionally, ErbB2 protein has been localised at the interface of GCs of activated growing follicles (Figure 3.6) and is absent from primordial follicles suggesting it may not be involved in the activation ‘trigger’. However, in light of the finding from Li-Ping et al., it would be interesting to examine the effect of ErbB2 inhibition on spontaneous follicle activation in vitro in the mouse. ErbB2 may have a role to play directly following either the release of an inhibitory signal or an activation signal to mediate GC cuboidalisation.

One technical consideration is that EGF ligand may not be penetrating the whole ovary in culture. However, this is unlikely as literature using similar whole rat ovary cultures have observed effects with EGF ligand added to culture medium (Kezele et al., 2002). Also work within the lab has found effect of other growth factor ligands, such as TGFβ2...
successfully elicit an effect on follicle activation when added to culture medium (Elizabeth Oliver, PhD thesis, 2017). Confirmation of EGF movement into the ovary could be achieved with the use of a fluorescently labelled EGF conjugate to examine penetration of EGF into the cultured ovaries.

5.3.2 EGF decreases average oocyte size in the neonatal ovary

Interestingly, although EGF did not affect the proportion of primordial follicle activation, it did decrease average oocyte size. There were several hypotheses why this may have occurred. The neonatal ovary experiences a significant loss of germ cells shortly after birth in the mouse (Kerr et al., 2006), with a 33% loss recorded between postnatal day 0 and 6 (Bristol-Gould et al., 2006). An interruption to this widespread follicle atresia would result in a larger pool of primordial follicles, lowering the average oocyte size. In the prepubertal cat ovary, EGF was shown to increase primordial follicle viability (Fujihara et al., 2014), hence EGF may also support the survival of small follicles otherwise destined to regress in the mouse ovary. However, average follicle counts per section revealed no significant difference in follicle number with EGF, suggesting it is not a lack of primordial follicle regression that decreases average oocyte size. Another explanation may be that EGF induces atresia of larger follicles, skewing the area average toward smaller oocyte sizes. However, there was no difference in staining for apoptosis marker cleaved caspase-3 in EGF treated ovaries.

Given that the decrease in average oocyte size is likely not associated with a change in follicle atresia, it would be interesting to examine the effect on EGF on factors known to control oocyte growth in vivo. For example, KL particularly the membrane bound KitL2 isoform has been shown to promote growth and survival of murine oocytes (Thomas et al., 2008, Packer et al., 1994). Oocytes from d12 mice cultured with KitL2-producing fibroblasts grew significantly larger, and suppression of KitL2 expression impaired oocyte growth. The effect of EGF on KL was not examined in the EGF quantitative RT-PCR screen (see Chapter 4) and would be interesting to investigate. The PI3K pathway activated downstream of KL, and its receptor c-Kit, plays a critical role in regulating oocyte growth (Liu et al., 2006). Additionally, downstream factors of the PI3K pathway such as the transcription factor Foxo3a are known to negatively regulate oocyte growth. Knockout of Foxo3a leads to overgrowth of oocytes in mice (Castrillon et al., 2003), and
expression of constitutively active Foxo2 leads to retardation of oocyte growth with a significant decrease in volume (Liu et al., 2007). Also the cdk inhibitor, p27, downstream of the PI3K pathway has been proposed as a negative regulator of oocyte growth, and p27 knockout mice have significantly enlarged oocytes (Liu et al., 2006). Although the previous results in this study have suggested the principal pathway stimulated by EGF is the MAPK cascade (see Chapter 4), EGF is known to activate the PI3K pathway and may play a role inhibiting oocyte growth. Interestingly, in the preantral follicle culture, EGF was shown to stimulate GC proliferation but did not stimulate a change in average oocyte size. The reason for the difference in average oocyte with EGF between the two culture systems is unclear and requires further investigation.

5.3.3 EGF has no visible effect on AMH protein expression in the neonatal ovary

In contrast to the findings in Chapter 4, which highlighted EGF as a negative regulator of both Amh mRNA and AMH protein in cultured preantral follicles, preliminary data showed no difference in AMH protein in the cultured neonatal ovary with EGF or AG1478. However, AMH protein in different follicles stages was not quantified and expression in stage-matched follicles should be compared in future studies. The lack of difference with AG1478 may again link to the low levels of EGFR protein in the d4 cultured ovary. Cultured ovaries are composed of primarily primordial, transitional and primary follicles with very few secondary follicles, it may be that the regulatory effect of EGF on AMH expression is only observed in later stage preantral follicles.

It would be interesting to investigate the effect of EGF treatment on older cultured ovaries that contain both primordial and larger preantral follicles, in which EGFR protein is highly expressed, to examine the effect on AMH expression and the subsequent effect of EGF on primordial follicle activation in the mature ovary. Recent work from the group has established an ovary fragment culture using d22-26 mice. These fragments contain follicles from the primordial to large antral stage of follicle development and could provide an appropriate culture system to address this hypothesis.
5.4 Conclusions

Taken together these results suggest that EGF and EGFR signalling does not play a role during primordial follicle activation in vitro, but may switch on at a later stage to regulate the progression of preantral follicle development.
Chapter 6: Effect of DHT on preantral follicle development

6.1 Introduction

Androgens, traditionally considered detrimental to ovarian function, are now known to play a crucial role in supporting normal preantral follicle development. The development of global and GC specific AR knockout mouse models revealed that AR deficient females have compromised fertility and reduced reproductive lifespans (Shiina et al., 2006, Walters et al., 2007, Hu et al., 2004). Importantly, ovaries obtained from these mice displayed altered growth and survival of follicles from the early stages of preantral development (Sen and Hammes, 2010, Walters et al., 2012). Various in vitro studies have also helped establish that androgens can stimulate growth and progression of early preantral follicles in fragments of bovine ovarian cortex (Yang and Fortune, 2006) and isolated mouse follicles (Murray et al., 1998).

Excessive androgen production, however, can be detrimental to preantral follicle development, and consequently impair fertility. Ovulatory abnormalities associated with disorders such as PCOS have been linked to dysregulation at the early stages of preantral follicle development (Franks et al., 2000, Franks et al., 2008). Ovarian cortical biopsies obtained from patients presenting with PCOS contain a significantly higher proportion of growing preantral follicles and fewer dormant primordial follicles (Webber et al., 2003). Although the mechanisms underlying the disruption in early follicle growth are uncertain, androgens are thought to play a central role. Prenatally androgenised ewes display disordered follicle development with accelerated progression of primordial follicles to the primary stage (Forsdike et al., 2007), displaying a reproductive phenotype in adulthood similar to that observed in women suffering from PCOS (Padmanabhan and Veiga-Lopez, 2013). It is therefore clear that a balance of intra-ovarian androgens must exist for normal follicle development.

This chapter takes a detailed look at the effect of androgens on preantral follicle development in the mouse. The androgen DHT was chosen owing to its inability to aromatise to oestrogen in culture, therefore the results obtained were attributed solely
to androgenic actions. Previous experimentation from within the lab determined from a
dose response with DHT (10 nM – 100 nM), that 10 nM elicited a near maximal effect on
follicle growth in vitro. The concentration 10nM DHT was chosen for all further studies,
as this concentration is similar to the approximate physiological concentration of DHT
reported in follicles (see Section 2.2.5.2).

The individual preantral follicle culture system was deemed the most appropriate to
investigate the action of DHT on preantral follicles. This established culture system
allows investigation into a specific window of follicle development, removing signalling
from surrounding stromal tissue giving a focussed examination of the preantral follicle
unit. A wide array of data from growth analysis to changes in gene and protein
expression and follicle survival rates can be obtained from this culture system. This
chapter also contains the development of a novel protocol designed to calculate the
proportion of nuclear vs. cytoplasmic AR staining in preantral follicles.

Some data presented in this Chapter contributed toward the paper published by Laird
et al., 2017.

6.1.1 Specific aims

- Localise AR protein in mouse ovarian tissue.
- Investigate the effect of DHT exposure on preantral follicle growth and GC
  proliferation in vitro.
- Explore the effect of DHT on preantral follicle survival.
- Examine the effect of DHT on gene and protein expression of key regulators of
  preantral follicle development.
- Investigate expression and nuclear localisation of AR protein following DHT
  treatment in preantral follicles in vitro.

6.2 Results

6.2.1 Localisation of AR protein in mouse ovarian tissue

Initially expression of AR protein in ovarian tissue was investigated in the d17 mouse
ovary. Immunofluorescence for AR was detected within dormant primordial follicles
and activated transitional follicles, with staining detected in the oocyte (Figure 6.1iv). Immunostaining for AR protein appeared strongest within the GCs of preantral follicles, specifically within follicles possessing one or two layers of GCs (Figure 6.1i-iii). AR protein was also investigated in the d28 mouse ovary to reveal that AR was present in the GCs of early antral follicles (Figure 6.1v). The IgG control showed minor background staining (Figure 6.1vi).

**Figure 6.1 AR protein immunolocalisation in mouse ovarian tissue.**
(i) AR staining (green) in a primary plus (1+) preantral follicle in a section from a d17 ovary, scale bar = 30 µm. (ii) Nuclei are labelled with DAPI (blue). (iii) Merged image of AR and DAPI staining in a preantral follicle. (iv) AR staining the oocyte of a primordial follicle (p), transitional follicle (t) and GCs of a multi-layered follicle (ML) in a section from a d17 ovary. (v) AR staining in antral follicle (An) in a section from a d28 ovary. Dashed line represents basal lamina. (vi) IgG Rabbit control of a section from a d17 ovary shows little background staining. Scale bars = 50 µm.

### 6.2.2 DHT stimulates preantral follicle growth through increased GC proliferation

To investigate the effect of androgens on early follicle development, mouse preantral follicles (75-130 µm diameter, 2-3 layers of GCs) were isolated from d16 mouse ovaries
and cultured individually in media supplemented with the potent androgen DHT. The 10 nM dose was determined by previous investigation in the lab (Laird et al., 2017). Follicles were photographed every 24 hours over the culture duration (Figure 6.2A). One interesting observation was that earlier than expected antrum formation was more prevalent in the DHT treated follicles. Initially this was believed to be an artefact caused by the increased follicle size with DHT, however the increase in antrum formation was not restricted to the largest follicles suggesting that another mechanism is in play (observations Kate Hardy, (Laird et al., 2017)). Exposure to DHT significantly increased preantral follicle growth above the vehicle control from 24 hours in culture (Figure 6.2B). Change in follicle size was justified to time 0 to account for variability in follicle size at isolation. The increase in overall follicle area in the DHT treated follicles was a result of the expansion in GC area rather than oocyte growth (Figure 6.2C).

**Figure 6.2 DHT stimulates preantral follicle growth in vitro.**
A) Representative images of follicles cultured in vehicle control or 10 nM DHT, scale bar = 50 µm. B) Percentage change in follicle area relative to 0 hour is shown. Values are mean ± SEM, statistical analysis between treatments were made within each time point by unpaired t-test where *** p < 0.001 (n = 6 ovaries). C) Change in area of oocyte and GCs (total follicle area - oocyte area) over culture period in follicles treated with DHT (10 nM). Values are mean ± SEM. Numbers bracketed represent number of follicles in each treatment group.
AR immunostaining was strongest in the early preantral follicles with 1 - 2 layers of GCs. Therefore, it was investigated if responsiveness to DHT changed with increasing follicle area at the start of culture. Follicles were grouped according to area at time 0, with small follicles classified as under 9000 µm² (diameter < 95 µm), medium follicles classed as between 9000 - 11000 µm² (diameter 95 - 105 µm), and large follicles over 11000 µm² (diameter > 105 µm). DHT had the most pronounced effect on smaller follicles, with the smallest follicles growing proportionately more than larger ones at 24, 48 and 72 hours, relative to 0 hours (Figure 6.3).

![Figure 6.3](image)

**Figure 6.3 Follicles of smaller initial size are more responsive to DHT in vitro.**
Follicles were cultured with vehicle control or 10 nM DHT for 72 hours and area analysis. Results were separated by follicle size at 0 hours. Initial sizes are separated by area at 0 hr: Small = < 9000 µm², Medium = 9000 - 11000 µm², Large = > 11000 µm². Percentage change in follicle area relative to 0 hour is shown. Values are mean ± SEM, statistical analysis between size groups was made within each time point by one-way ANOVA and Tukey’s multiple comparison test. Statistical results shown are comparisons between small vs. large, where * p < 0.05, ** p < 0.01 (n = 6 ovaries). Numbers bracketed represent number of follicles in each size group.

The mitotic activity of GCs was assessed in DHT treated follicles following the expansion observed in GC area. Immunofluorescence staining for cell cycle marker Ki67 protein was higher within the GCs of the DHT treated follicles compared to control (Figure 6.4A). The proportion of GC nuclei stained positively for Ki67 was calculated and tested significantly higher at both 24 and 72 hours with DHT treatment (Figure 6.4B).

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Figure 6.4 DHT stimulates GC proliferation in preantral follicles in vitro.
A) Follicles cultured in vehicle control or DHT (10 nM) were fixed and stained for Ki67 (green) and counterstained with nuclei label DAPI (blue), representative images at 24 hours and 72 hours are shown, scale bar = 50 µm. B) Proportion of Ki67-positively stained nuclei counted in DHT treated follicles at 24 hours (n = 11 - 15 follicles) and 72 hours (n = 21 - 28 follicles) is shown. Bars represent mean ± SEM, compared using unpaired t-test where ** p < 0.01.

AR is a nuclear receptor and acts classically as a ligand-activated transcription factor. The increase observed in GC proliferation with DHT prompted the investigation into the effect of DHT on genes involved in cell cycle progression. Classical signalling of AR stimulates changes in gene expression detected within a few hours post-stimulation. Preantral follicles are significantly larger with DHT treatment at 24 hours in culture, suggesting AR is likely activating changes in gene expression before this first 24 hour time point. Three time points leading to 24 hours were chosen to assess the effect of DHT on gene expression in cultured follicle. Preantral follicles were cultured individually in media supplemented with vehicle control or 10 nM DHT for 6, 12 or 24 hours and collected for RNA extraction. Transcript levels of one regulator of the cell cycle, Ccnd2, was decreased at 24 hours, while Cdkn1b was depressed at both 12 and 24 hours compared to control (Figure 6.5). Myc (c-Myc) encodes a transcription factor that plays a role in cell cycle progression, apoptosis and cellular transformation. Levels of Myc were significantly elevated with DHT treatment at 24 hours.
Figure 6.5 Effect of DHT on gene expression of cell cycle regulators in cultured preantral follicles.

Preantral follicles cultured with or without DHT (10 nM) for 6, 12 or 24 hours. Gene expression data is displayed as fold change with DHT relative to control within each time point. Bars represent mean ± SEM (n = 6 ovaries, 6 follicles from same ovary pooled). Statistical analysis between control vs. DHT by unpaired t-test within time points, where * p < 0.05, ** p < 0.01, *** p < 0.001.

6.2.3 DHT does not increase follicle survival in culture

There have been multiple reports that DHT has anti-apoptotic properties in the ovary. Although follicles appeared morphologically healthy over the culture period, health was confirmed by quantifying levels of follicle atresia in the cultured follicles by double immunostaining for apoptosis markers TUNEL and cleaved caspase-3 at 24 hours and at the completion of culture at 72 hours (Figure 6.6A). There was no difference observed in the proportion of positively stained GC nuclei for TUNEL in control and DHT treated follicles (Figure 6.6B). Few cells stained positively for cleaved caspase-3 in control and DHT treated follicles with no difference observed between the treatment groups.
Figure 6.6 DHT has no effect on apoptosis markers TUNEL and cleaved caspase-3 in preantral follicles in vitro.

A) Follicles cultured in vehicle control or DHT (10 nM) were fixed and stained for TUNEL (green) and cleaved caspase-3 (red), counterstained with DAPI (blue), representative images at 24 hours and 72 hours are shown, scale bar = 25 µm. B) Proportion of nuclei stained positively for TUNEL or cleaved caspase-3 in control or DHT treated follicles at 24 hours (n = 9 - 10 follicles) and 72 hours (n = 9 - 21 follicles). Bars shown are medians, statistically analysed using Mann Whitney test.

Although there appeared no difference in TUNEL and cleaved caspase-3 staining with DHT treatment, levels of cell death varied considerably. Therefore, mRNA levels of genes associated with apoptosis were also investigated with DHT. Transcript levels of pro-apoptosis related gene Bad remained unchanged in DHT treated follicles, as did levels of the anti-apoptotic gene Bcl2 (Figure 6.7). A drawback of this analysis is that cultured follicles were pooled to obtain sufficient quantities of RNA for quantitative RT-PCR. Single follicle PCR has been performed successfully in our lab and could be used in further studies to increase accuracy of data.
Figure 6.7 Effect of DHT on gene expression of apoptosis regulators in cultured preantral follicles.
Preantral follicles cultured with or without DHT (10 nM) for 6, 12 or 24 hours. Gene expression data is displayed as fold change with DHT relative to control within each time point. Bars represent mean ± SEM (n = 6 ovaries, 6 follicles from same ovary pooled).
Statistical analysis between control vs DHT by unpaired t-test within time points.

One limitation to this analysis is that only morphologically healthy follicles were collected at the completion of culture for molecular and immunohistochemical analysis. Therefore, another investigation was undertaken examining the survival rates of follicles from multiple cultures with DHT treatment. Follicles from 3 separate follicle culture experiments were grouped by ovary (n = 17) and percentage of follicles that were classed as ‘morphologically healthy’ at the end of culture (using photographs taken at 72 hours) was assessed. Follicles were classed as unhealthy if (i) the oocyte was not centrally located, (ii) the oocyte had been excluded, (iii) the oocyte was misshapen or (iv) the GCs had darkened indicative of atresia. There was no significant difference in follicle survival rates (classed as ‘healthy’ at the end of culture) with the supplementation of culture medium with DHT in vitro (Figure 6.8).
Figure 6.8 DHT has no effect on follicle survival rates over culture. Morphology of follicles cultured in vehicle control or DHT (10 nM) was assessed at the completion of culture at 72 hours. Follicles categorised as ‘healthy’ were taken for further study, while follicles categorised as ‘unhealthy’ were discarded. Percentage survival of follicles (percentage classed as healthy at 72 hours) was calculated per ovary (n = 17 ovaries, 6 - 8 follicles in each treatment per ovary). Bars shown are mean ± SEM, statistically analysed by unpaired t-test.

6.2.4 DHT alters mRNA and protein expression of key regulators of follicle development

The effect of DHT on the mRNA levels of key regulators of preantral follicle development was investigated. Some studies have proposed that androgens positively regulate AMH expression in the ovary, as serum levels of testosterone and AMH are tightly correlated in women with PCOS (Pigny et al., 2003). However, DHT treatment significantly decreased Amh mRNA levels in cultured mouse preantral follicle (Figure 6.9). Interestingly, DHT treatment also caused a suppression of its own receptor Ar mRNA levels. Our group and others have shown a stimulatory effect of androgens on Fshr mRNA in small developing preantral follicles (Laird et al., 2017, Weil et al., 1999), so Fshr was included in this study as a positive control. Finally, gene expression of steroidogenic enzymes, Star and Cyp11a1, which are the rate limiting enzymes for all steroid synthesis, was investigated. Star, which plays a key role in making cholesterol available for steroid synthesis, was significantly elevated, while there was no change reported in the cholesterol side-chain cleavage enzyme Cyp11a1.
Figure 6.9 Effect of DHT on gene expression of key regulators of preantral follicle development.
Preantral follicles cultured with or without DHT (10 nM) for 24 hours. Gene expression data is displayed as fold change with DHT relative to control. Bars represent mean ± SEM (n = 5 ovaries, approx. 15 follicles from same ovary pooled). Statistical analysis between control vs DHT by unpaired t-test within time points, where ** p < 0.01, *** p < 0.001.

The decrease in Amh mRNA was investigated further by immunolocalisation of AMH protein in preantral follicles cultured with DHT (Figure 6.10A). Unexpectedly, AMH protein was significantly elevated with DHT at 24 hours compared to vehicle control (Figure 6.10B), in contrast with the results seen at the mRNA level. Interestingly, the localisation of AMH protein also appeared to change upon DHT exposure, becoming stronger in the GCs closest to the oocyte. By 72 hours however, there was no difference in AMH protein between the two treatments.
Figure 6.10 Immunolocalisation and quantification of AMH protein in preantral follicles treated with DHT.
Preantral follicles cultured with DHT (10 nM) or vehicle control for 24 or 72 hours. A) Sections of follicles immunolabelled for AMH (green) and counterstained with DAPI-labelled nuclei (blue). Scale bar = 50 µm. B) Quantification of AMH using ImageJ, calculated as percentage positive staining above a threshold in the GC compartment at 24 hours (n = 11 - 15 follicles) and 72 hours (n = 5 - 12 follicles). Bars represent mean ± SEM, statistically analysed by unpaired t-test within time points, where **** p < 0.0001.

6.2.5 DHT increases AR protein expression and nuclear localisation
DHT can positively regulate the protein expression of its own receptor in other tissue types (Krongrad et al., 1991), rendering cells more sensitive to androgen signalling. In cultured mouse preantral follicles, protein expression of AR was dramatically increased at both 24 and 72 hours (Figure 6.11A-B).
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**Figure 6.11 Immunolocalisation and quantification of AR with DHT in preantral follicles in vitro.**

Preantral follicles cultured with DHT (10 nM) or vehicle control for 24 or 72 hours. A) Sections of follicles immunolabelled for AR (green) and counterstained with DAPI-labelled nuclei (blue). Scale bar = 50 µm. B) Quantification of AR using ImageJ, calculated as percentage positive staining above a threshold in the GC compartment at 24 hours (n = 8 - 10 follicles) and 72 hours (n = 9 - 10 follicles). Bars represent mean ± SEM, statistically analysed by unpaired t-test within time points, where **** p < 0.0001.

Upon closer examination of AR protein expression in cultured follicles it was evident that AR staining appeared more nuclear in the DHT treated follicles. In order to accurately quantify this observation, a protocol calculating the degree of AR staining co-localised with nuclear DAPI was developed (Figure 6.12). Follicles were immunostained for AR protein and counter-stained with the nuclear label DAPI. Images were Red-Green-Blue (RGB) stacked using ImageJ software and the DAPI (blue) channel isolated. An appropriate threshold was set for DAPI staining and nuclei were outlined using ‘Analyse Particles’ function. The location and size of the DAPI particles was recorded using an ROI manager. The original image was re-opened on ImageJ and RGB stacked once again, but this time the AR (green) channel was isolated. An appropriate threshold was set for AR staining and total area above the threshold (represented as pixels) was measured within the GC compartment. The DAPI defined particles were then overlaid from the ROI manager onto the AR channel. The amount of staining (represented as pixels) within the DAPI particles was measured. From these two values, the proportion of AR staining in the nucleus (i.e. co-localised within DAPI particles) could then be calculated.
Figure 6.12 Protocol for quantifying proportion of AR immunostaining in nucleus. Preantral follicles were immunolabelled for AR (green) and counterstained with DAPI-labelled nuclei (blue). Merged images were RGB stacked on ImageJ, and DAPI stained nuclei were isolated and outlined. The DAPI stained nuclei outlines were overlaid on the RGB stacked AR channel. AR staining above a threshold within the nuclei outline was measured, as was total AR staining. Percentage staining co-localised with nuclear DAPI, and not co-localised (cytoplasmic AR) could then be calculated.

Quantification of nuclear AR confirmed what was seen visually, that AR staining was significantly more nuclear in the DHT treated follicles at both 24 and 72 hours compared to vehicle control (Figure 6.13).
Figure 6.13 Nuclear localisation of AR is increased with DHT in preantral follicles in vitro.

Preantral follicles were cultured with vehicle control or DHT (10 nM) for 24 or 72 hours, then immunolabelled for AR and counterstained with DAPI-labelled nuclei. Percentage of AR localised to the nucleus was calculated. Data shown is mean (horizontal line) ± SEM and individual data points at 24 hours (n = 7 - 10 follicles) and 72 hours (n = 8 - 10 follicles). Statistically analysed by unpaired t-test where, **** p < 0.0001.

6.3 Discussion

6.3.1 AR protein is expressed at all stages of preantral follicle development

AR protein expression has been detected throughout the mammalian HPG axis, identified in the brain as well as ovarian tissue. Distinct spatial and temporal AR mRNA and protein expression has been described previously in the ovary, giving an indication of the relative importance of androgen signalling throughout follicular development. In the present study AR protein was detected in the ooplasm of the murine primordial follicle. This result is in contradiction to the earlier literature which reported AR absent in primordial follicles in rat (Szoltys and Slomczynska, 2000), cow (Hampton et al., 2004), sheep (Juengel et al., 2006), primate (Hild-Petito et al., 1991) and human (Rice et al., 2007, Suzuki et al., 1994) ovary. This contrasting result may be due to species variability. However, androgen treatment has been shown to stimulate primordial follicle activation in the neonatal mouse ovary in vitro, which could provide functional evidence for AR expression in mouse primordial follicles (Yang et al., 2010). It is widely agreed in the literature that AR is present in activated primary follicles, in the rat...
(Szoltys and Slomczynska, 2000), cow (Hampton et al., 2004, Salvetti et al., 2012), sheep (Juengel et al., 2006), primate (Hild-Petito et al., 1991) and human (Rice et al., 2007) ovary. The current study found that AR staining was strongest in the preantral follicles with 1 to 2 layers of GCs. AR protein has been found in other model species to be the most abundant in preantral and early antral follicles in primate ovary (Hillier et al., 1997), and strongest at the preantral stage in rodent ovaries (Lenie and Smitz, 2009, Tetsuka et al., 1995). The present study also briefly examined the expression of AR in small antral follicles within a d28 mouse ovary. Similar to previous reports, AR staining was strong in the GCs of antral follicles. AR expression is proposed to weaken in GCs as follicles progress to the pre-ovulatory stage (Hillier et al., 1997), with a gradient of AR staining stronger in the cumulus cells closest to the oocyte and becoming weaker in the mural GCs (Szoltys and Slomczynska, 2000). This later stage of follicle development was not examined in the present study however these studies indicate the temporal importance of androgen signalling during the preantral stages of follicle development.

In the present study, AR protein was expressed in the oocyte and theca cells of preantral follicles as well as the surrounding stroma, however the strongest expression was observed within the GC compartment. This is in agreement with the expression pattern reported in the primate ovary, which found most abundant AR expression in the GCs of healthy preantral follicles, with lesser expression observed in the thecal and stromal cells (Weil et al., 1998, Hillier et al., 1997). The spatial expression observed in the mouse ovary infers that GCs are the primary recipients of androgen signalling within the developing preantral follicle. Indeed, AR knockout mice have shown the most severe reproductive phenotype is displayed in GC-specific ARKO mice, in comparison to oocyte- and theca cell-specific ARKO mice which have normal fertility (Sen and Hammes, 2010).

**6.3.2 DHT stimulates preantral follicle growth in vitro**

Supplementation of culture medium with DHT significantly increased preantral follicle size compared to the vehicle control in vitro. DHT is a non-aromatisable androgen, meaning this stimulation can be confidently attributed to an androgenic effect and not a consequence of aromatisation to oestrogen and stimulation of the ER. Androgen treatment has been previously reported to enhance follicle progression and growth of
mouse preantral follicles *in vitro*. Preantral follicles from immature mice increase in diameter after 4 days in culture with testosterone and DHT (Wang et al., 2001). Furthermore, cultures supplemented with an anti-androgen or AR antagonist bicalutamide significantly suppress growth of whole murine follicles, an effect which was reversed by the addition of androstenedione to the medium (Murray et al., 1998). Similarly, evidence from other model species, such as cultured bovine ovarian cortex, has shown that testosterone stimulates the transition of primary to secondary follicles (Yang and Fortune, 2006). In studies using testosterone as the primary androgen added to culture it is unclear if the effects reported are consequences of direct androgen action via AR or due to aromatization of androgens to oestrogen. However, the in the case of the bovine ovarian cortex, the stimulatory effect was blocked by the anti-androgen flutamide indicating an androgenic effect. There is also evidence provided by *in vivo* studies that have supported the role of androgens in promoting preantral follicle progression. Administration of either testosterone or DHT increases the number of growing preantral and small antral follicles in the primate ovary (Vendola et al., 1998) and in the ovary of prenatally androgenised ewes (Smith et al., 2009).

The most pronounced effect of DHT was on the smallest follicles isolated. This result fits with the observation that AR protein expression was strongest in the early preantral follicles with 1 – 2 layers of GCs. However, another consideration is that once follicles surpass a certain size they require FSH to develop further. Our group has shown that mouse preantral follicles in culture become increasing responsive to FSH as they grow (Hardy et al., 2017). As FSH is lacking from the culture medium this may restrict the growth of larger follicles *in vitro*.

The increase in preantral follicle size was accompanied by an increase in the proportion of Ki67-positive nuclei in the GCs of preantral follicle, indicating that androgens promote follicle progression through stimulation of GC proliferation. Additionally, there was no change in oocyte size over the culture period but a significant expansion in the GC area. Increase in GC proliferation with DHT has been detected previously through enhanced immunofluorescence staining of 5-bromo-2’-deoxyuridine within the nuclei of GCs (Wang et al., 2001). Androgens may stimulate GC proliferation through AR binding to DNA to regulate the transcription of genes involved in mitogenesis. However,
androgens have also been shown to indirectly augment the mitogenic effects of other factors, such as oocyte-secreted factors and GDF9 in porcine GCs (Hickey et al., 2005), and IGF1 in porcine preantral follicles (Hickey et al., 2004). The effect of DHT on growth factor signalling is explored further in Chapter 7.

There have so far been few proven AR target genes identified in the preantral follicle. DHT treatment altered mRNA levels of cell cycle regulators \textit{Ccnd2} and \textit{Cdkn1b} in cultured preantral follicles at 24 and 12 hours respectively. It remains unclear if this decrease is a direct effect of AR binding to the regulatory region of the gene and repressing transcription or through indirect mechanisms. Cyclin D2, as mentioned in Chapter 4, plays an essential role in gonadal cell proliferation and cyclin D2 KO female mice are sterile due to restricted GC proliferation (Sicinski et al., 1996). The activity of cyclin D2 is required for cell cycle G1/S transition, so it was unexpected that this gene was repressed with DHT treatment. However, the activity and degradation of cyclin D2 protein may be more important than its transcription. DHT has been shown previously to decrease mRNA levels of cyclin D2 in GCs from rats administered DHT (Pradeep et al., 2002), however this was accompanied by a decrease in GC proliferation, the opposite of which was reported in the current study. The decrease observed in \textit{Cdkn1b} gene expression with DHT may encourage cell cycle progression at G1, as \textit{Cdkn1b} encodes a cell cycle inhibitor (p27Kip1) that binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes. Regulation of P27Kip1 expression is critical to fertility, p27 knockout mice exhibit impaired ovulation along with premature and over-activation of the follicular pool resulting in premature ovarian failure (Rajareddy et al., 2007). AR mediated repression of \textit{Cdkn1b} may therefore stimulate the elevation in GC proliferation and follicle growth seen with DHT.

Myc mRNA and protein has been detected in GCs of preantral follicles, but not antral follicles in porcine (Sato et al., 1994) and human ovaries (Li et al., 1994), suggesting a stage-specific role for Myc in GC proliferation. Induction of Myc expression has been shown to promote GC proliferation and rescue mitotic arrest induced by Notch signalling inhibitors L-658,458 and DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-buty] ester) in cultured mouse primary follicles (Zhang et al., 2011). In the present study, DHT treatment induced \textit{Myc} transcription at 24 hours. The
transcription factor encoded by \textit{Myc} activates expression of genes driving cellular proliferation, and may be partly responsible for the mitogenic response in GCs with DHT. Additionally, the stage specific expression of Myc may also contribute to the variation in response to DHT observed in follicles of different sizes. A recent publication by Barfeld showed that overexpression of c-Myc antagonises AR activity and transcriptional output (Barfeld et al., 2017). In a PCa cell line model, c-Myc overexpression reduced transcription of AR target genes, partially reprogrammed AR chromatin occupancy and altered histone mark distribution. The DHT induced increase in Myc expression may lead to deregulation of AR transcriptional activity, providing a mechanism of negative feedback in androgen signalling within the follicle.

\textbf{6.3.3 DHT stimulates premature antrum formation \textit{in vitro}}

An interesting observation noted during the culture was the increased prevalence in premature antrum formation in the DHT treated follicles. This may be attributed to the accelerated growth observed in preantral follicles exposed to DHT. However, there was no significant difference in the diameter between follicles with and without an antrum (Laird et al., 2017), suggesting another mechanism may be responsible. Aquaporins are proteins in the cell membrane which facilitate water transport into and out of the cells and have been proposed to play a role in the accumulation of fluid during the formation of the follicular antrum (Huang et al., 2006). Fluid is likely derived from blood flowing through the thecal capillaries and has to traverse the follicular basal lamina as well as GC membranes through transcellular mechanisms to expand the antral cavity of the developing follicle (Rodgers and Irving-Rodgers, 2010). Aquaporins 7, 8 and 9 have been detected in rat GCs (McConnell et al., 2002) and aquaporins 5 and 9 have been detected in porcine GCs (Skowronski et al., 2009). Aquaporins may be androgen responsive, as testosterone has been shown to influence water transport in porcine GCs, significantly increasing the swelling of GCs cells when moved from isotonic to hypotonic medium over control (Grzesiak et al., 2013). This increase in water permeability caused by testosterone was abolished with the addition of the antiandrogen hydroxyflutamide. In other cell types, such as prostate and epididymal epithelium, androgens have also been shown to regulate the expression of aquaporin 9 (Wang et al., 2008, Pastor-Soler et al., 2002). Additionally, \textit{in vivo} studies have shown that administration of the antiandrogen flutamide during gestation or neonatally in pigs causes a decrease in both
mRNA and protein levels of aquaporin 5 in both preantral and antral follicles (Grzesiak et al., 2016), indicating that androgens regulate aquaporin expression in follicles in vivo.

These studies together indicate that aquaporin expression is responsive to androgen signalling. An increase in aquaporin expression in DHT treated follicles may explain the increased prevalence of antrum formation by facilitating the accumulation of water from the surrounding medium, and should be investigated in future studies. One conflicting study to this hypothesis examined expression of aquaporin-9 in human GCs, found a negative correlation between hyperandrogenism in follicular fluid and aquaporin-9 mRNA levels in GCs from patients with PCOS (Qu et al., 2010). Additionally, in vitro treatment with DHT decreased aquaporin-9 mRNA and protein levels in control GCs. However, these studies are undertaken at a much later stage in follicle development, and the granulosa luteal cells obtained from women undergoing oocyte retrieval for IVF are biologically dissimilar to GCs from preantral follicles.

6.3.4 DHT does not promote preantral follicle survival in vitro

Androgens have been associated with increased preantral follicle survival in various in vitro and in vivo studies. Additionally, follicles from women with typically high circulating androgens, diagnosed with polycystic ovaries, demonstrate decreased rates of atresia in cultured ovarian biopsies compared to control (Webber et al., 2007). Androgens serve as essential substrate for the survival factor oestradiol (Hillier et al., 1994, Harman et al., 1975) but signalling through AR has also been shown to have direct effects on follicle health. Follicle atresia is elevated in all ARKO mouse models that abolish AR signalling in the ovary (Hu et al., 2004, Shiina et al., 2006, Walters et al., 2007). In the GC-specific AR knock out model, the number of unhealthy follicles (classified as unhealthy if they contained a degenerate oocyte and/or >10% of the granulosa cells were pyknotic in appearance) was increased 81% compared to controls at 6 months of age leading to premature reduction in female fecundity (Walters et al., 2007). Also administration of testosterone or DHT to primates for 3 – 10 days reduces proportion of apoptotic GCs and follicle atresia in vivo (Vendola et al., 1998). Although the mechanisms underlying decreased atresia remain undefined, DHT has recently been found to attenuate follicular apoptosis in vitro by enhancing GC expression of the anti-

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apoptotic microRNA, microRNA125b, thereby suppressing expression of pro-apoptotic proteins such as BAK1, BMX, BMF and TRP53 (Sen et al., 2014).

The current study, however, found no difference in protein expression of apoptosis markers TUNEL or cleaved caspase-3 in GCs of cultured preantral follicles with DHT. The variability in the level of immunostaining was high between individual follicles. Therefore, quantitative RT-PCR for levels of pro-apoptotic Bad and anti-apoptotic Bcl-2 mRNA was performed, however no difference between treatments was observed. One possibility is that a longer culture period may reveal differences in apoptotic markers. A limitation to this analysis was that only follicles which were morphologically healthy were collected for further analysis, also follicles were pooled for quantitative RT-PCR which may have masked subtle changes in individual follicles. To address this, follicle survival rates were measured across various cultures with DHT but no difference between treatments was detected.

The reason behind the lack of change in follicle survival is unclear but may be related to the dose of androgen used. High levels of androgens have been shown to enhance GC apoptosis. In hypophysectomised immature female rats androgen treatment negatively correlates with ovarian weight indicative of increased follicular atresia (Hillier and Ross, 1979). Testosterone treatment of rats has also been shown to enhance GC apoptosis of antral and preantral follicles and increase ovarian apoptotic DNA fragmentation (Billig et al., 1993). Furthermore, ovarian serial sections of hypophysectomised rats treated with DHT or testosterone revealed the presence of pyknotic nuclei suggestive of follicle atresia was increased with androgen treatment, but interestingly only in ‘large follicles’ with no change in atresia seen in follicles measuring smaller than 150 µm in diameter (Azzolin and Saiduddin, 1983). The androgen DHT, used in the current study, is three to ten times more potent than testosterone. It is possible that the 10nM dose of DHT is high enough to offset any positive influence on follicle survival. The experiment may be repeated in the future with a lower dose of DHT to investigate this hypothesis further.
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It is likely that a balance of AR signalling is necessary to maintain follicle health. However, the majority of research to date indicates that moderate doses of androgens have a predominately positive role to play in maintaining preantral follicular survival.

6.3.5 DHT alters expression of FSHR, AMH and STAR in preantral follicles

The stimulatory effect of androgens on FSHR have been reported in small developing preantral follicles in vivo (Weil et al., 1999), as well as by our group in preantral mouse follicles in vitro (Laird et al., 2017). The increase in FSH sensitivity likely contributes to the synergistic effect of combined FSH and androgen treatment on preantral follicle growth in culture (Wang et al., 2001, Laird et al., 2017), and may provide another mechanism by which androgens stimulate follicle development in vivo. Accordingly, GCs collected from women with PCOS during oocyte retrieval express significantly higher FSHR mRNA levels than GCs from control women (Catteau-Jonard et al., 2008).

Positive correlation between serum levels of testosterone and AMH have been reported in women with PCOS (Pigny et al., 2003), as well as positive correlations between AMH and AR expression in individual follicles from PCOS and control women (Catteau-Jonard et al., 2008), causing some to postulate that excess androgens may regulate AMH production within the ovary. However, work from our group has shown that AMH protein expression was actually lower in primordial and transitional follicles in ovarian biopsies taken from anovulatory women with polycystic ovaries than control (Stubbs et al., 2005), and evidence for a direct regulatory mechanism of androgen on AMH production is lacking. In vitro cultures of bovine small follicles display a down-regulation of AMH mRNA and protein expression in GCs from small follicles with testosterone (Crisosto et al., 2009). Also in vitro studies conducted in our lab, examining the effect of androgen exposure on isolated preantral follicles, found a decrease in Amh mRNA levels in DHT treated follicles at 24 hours (Laird et al., 2017). These studies are in agreement with in vivo studies in sheep prenatally exposed to testosterone, which reported reduced AMH expression in large preantral follicles in 10- and 21-month-old ewes (Veiga-Lopez et al., 2012). Decreased AMH expression associated with androgens may contribute to disordered early follicle development in PCOS.
It is unclear why, in the present study, the decrease observed in *Amh* in DHT treated follicles was not translated into decreased AMH protein, which in fact was significantly increased with DHT treatment. AMH protein is largely regulated by factors derived in the oocyte, illustrated through co-culture of isolated oocytes and GCs, which resulted in over a 2-fold increase in AMH expression (Salmon et al., 2004). Also work from our lab has shown that removal of oocyte specific factors through oocytectomy of preantral follicles dramatically reduces *Amh* mRNA (Mora and Fenwick, unpublished observations). The increase observed in AMH protein in the present study appeared localised to the GCs surrounding the oocyte. One hypothesis is that DHT treatment alters expression of oocyte derived factors and indirectly alters AMH protein expression. Indeed, studies carried out in the lab in parallel showed that DHT treatment significantly decreased oocyte specific *Bmp15* mRNA levels in cultured mouse preantral follicles at 24 hours (Laird et al., 2017). BMP15 has been shown to stimulate AMH mRNA and protein expression in humans GLs (Ogura-Nose et al., 2012), hence reduction of BMP15 levels will contribute to reduced AMH levels. It would be interesting in future studies to investigate the effects of DHT on other oocyte specific growth factors during culture.

Androgen signalling has been linked to altered expression of steroidogenic enzymes in many of the ARKO animal models, reviewed by Walters (Walters and Handelsman, 2017). Star is considered one of the rate limiting steps in all steroid synthesis in the ovary, regulating cholesterol transfer in the mitochondria. An increase in Star expression may therefore translate to an increase in steroid synthesis within the developing follicle. Interestingly, one of the characteristics of the polycystic ovary is perturbed steroidogenesis. Star is also regulated by oocyte secreted factors such as BMP15 which has been shown to decrease Star mRNA levels in human GLs (Ogura-Nose et al., 2012). Therefore, a decrease in BMP15 expression by androgens (Laird et al., 2017), may contribute to the increase seen in Star.

### 6.3.6 DHT alters AR protein expression and localisation in preantral follicles

DHT treatment dramatically increased AR protein expression in cultured preantral follicles. Increase in the expression of AR protein, may be through various means such
as altered transcription, translation or post-translational modifications. The transcriptional regulation of AR is controlled by various transcription factors which bind to the DNA in a sequence-specific manner to positively or negatively affect gene expression. Binding sites for several transcription factors have been mapped within the Ar gene, including CREB, Myc, c-Jun, Sp1, p53, and Foxo3a (Shiota et al., 2011). DHT has been shown to positively regulate the transcription of AR through the activation of Myc in LNCaP cells (Lee et al., 2009). Another study found that treatment of LNCaP cells with androgen did not increase AR mRNA but increased AR protein 2-fold following 24 hours incubation. The authors credited this increase to ligand-induced changes in AR protein stability and/or increased translational efficiency of AR mRNA (Krongrad et al., 1991). The current study found that Ar mRNA was decreased at 24 hours in mouse preantral follicles, which may be a result of a negative feedback mechanism, caused by the substantial increase in AR protein expression. Similarly in human prostate cancer cells, LNCaP, and in various rat tissues, AR mRNA has been shown to decrease below control levels after androgen stimulation and increase two- to ten-fold with androgen withdrawal (Quarmby et al., 1990, Krongrad et al., 1991). Additionally, in vivo rat models have shown that AR mRNA levels in various tissues including the prostate and seminal vesicles increased within 24 hours after castration, and administration of pharmacological doses of testosterone to castrated animals resulted in a reduction in AR mRNA levels (Shan et al., 1990, Quarmby et al., 1990). It has been shown that the androgen-induced negative feedback on AR mRNA levels results from an inhibition in AR transcription in LNCaP cells (Blok et al., 1992). A study by Cai et al. in 2011 investigated the mechanism underlying this AR mRNA transcriptional repression and found that increased androgen levels encouraged AR binding and recruitment of the cofactor lysine-specific demethylase 1 (LSD1) and H3K4me1,2 demethylation resulting in the repression of AR gene expression (Cai et al., 2011).

Positive feedback loops of AR protein expression have been reported previously in testosterone exposed mouse. Mice administered testosterone for 7 days displayed significantly increased AR protein expression in the stroma of ovarian cortex compared to control, shown through immunohistochemistry and western blot analysis (Yang et al., 2015). Furthermore, AR expression was reported as significantly increased in the GCs of testosterone treated rhesus monkeys from preantral to large antral follicle stage (Weil
et al., 1998). One of the clinical hallmarks of PCOS is hyperandrogenism, and GCs collected from women with PCOS undergoing oocyte retrieval during IVF show an overexpression of AR mRNA levels (Catteau-Jonard et al., 2008), presenting another scenario by which high testosterone may regulate AR expression in GCs. Androgen exposure to developing preantral follicles may therefore create a positive feedback mechanism, in which androgens increase expression of their own receptor, increasing sensitivity to androgen exposure supporting follicular development. This result may explain the sustained growth trajectory of preantral follicles exposed to DHT, increasing growth significantly at 48 and 72 hours.

AR is a ligand activated nuclear receptor, and binding of androgen to AR results in translocation to the cell nucleus. Following DHT treatment of mouse preantral follicles, AR immunostaining was significantly more nuclear than in control follicles, demonstrating that AR is acting through genomic mechanisms to regulate gene transcription. However, there remained AR staining within the cytoplasm of the GCs following DHT treatment. Recent evidence has shown that ARs have a second mode of action, signalling within the cytoplasm to induce rapid changes in cell function and indirectly leading to changes in gene and protein expression. Activation of AR in the cytoplasm will be explored further in Chapter 8.

6.4 Conclusions

Androgens play a critical role in regulating preantral follicle development in the mouse. AR protein is present at all stages of preantral follicle development, peaking in the early preantral follicle. Similar to previous studies, isolated preantral follicles display amplified GC proliferation and follicle growth in response to androgens. Additionally, androgen exposure positively regulates expression of its own receptor, producing a positive feedback loop further increasing sensitivity and response to androgens. Increased nuclear localisation of AR was observed in the GCs of DHT treated follicles, showing that AR translocates and acts within the nucleus upon androgen exposure. The expression of key regulators of preantral follicle development, such as FSHR and AMH, were altered in androgen exposed follicles and may provide insights into the disordered early follicle development seen in women with PCOS.
Chapter 7 Crosstalk between androgens and the EGF Family

7.1 Introduction

The molecular mechanisms underlying androgenic regulation of preantral follicle development remain unclear. This chapter presents the hypothesis that androgens may be acting in part through modulation of growth factor signalling. The important role of the EGF family of growth factors during preantral follicle development has been characterised in Chapters 3-5. Furthermore, the ErbB receptors have been highlighted in published GWAS studies as potentially altered in women with typically high levels of circulating androgens and diagnosed with PCOS (Day et al., 2015). Uncovering evidence for potential crosstalk between androgen signalling and the EGF family was the focus of further investigation.

Androgens have been reported to interact with key growth factors in the ovary that mediate follicle development such as IGF in both *in vitro* and *in vivo* models, increasing expression and activation of the IGF1R (Hickey et al., 2004, Vendola et al., 1999). The first section of this chapter will examine the effect of androgens on the expression of the EGF-like ligands and ErbB receptors in mouse preantral follicles. Alterations in the expression of growth factor receptors may enhance or diminish the sensitivity of follicles to stimulatory growth factor signalling. Additionally, preantral follicle cultures exposed to a combination of DHT and EGF treatment were used to explore a potential synergistic effect of the two ligands.

In order to investigate if activity of the ErbBs or their downstream signalling pathways was involved in androgen stimulated follicle development, preantral follicles were cultured with DHT in combination with various ErbB pathway inhibitors and follicle growth was measured (Figure 7.1).
Figure 7.1 Inhibitors used to investigate crosstalk between AR and EGF like family signalling.

Dihydrotestosterone (DHT) binds to and activates androgen receptor (AR). AR can act within the cytoplasm (dotted grey line) or translocate to the nucleus to regulate gene transcription (complete black line) to increase proliferation and preantral follicle growth. To investigate a potential crosstalk with the EGF signalling family or their downstream second messengers, inhibitors of EGFR (AG1478), ErbB2 (ErbB2 inhibitor ii), MEK1/2 (U0126) and PI3K (LY294002) were added along with DHT to culture medium and follicle growth measured. Turquoise receptor represents ErbB dimerisation partner. HSP, heat shock protein; EGF, epidermal growth factor; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated protein kinases; PI3K, phosphatidylinositol 3-kinase.

Finally, crosstalk between androgens and EGF family signalling was explored in human GL cells. The lab is fortunate to have access to samples from control women and women diagnosed with PCOS, in the form of GL cells collected during oocyte retrieval from...
patients undergoing IVF treatment. One of the hallmarks of PCOS is excessive androgen production. These samples provide a valuable pathophysiological model to examine biological differences in follicular cells that have developed in a typically high androgenic ovarian environment. GL cells were used to compare ErbB expression in control and PCOS ovaries.

7.1.1 Specific aims

- To investigate the effect of DHT on EGF-like ligand and ErbB receptor expression in cultured mouse preantral follicles.
- To explore the importance of ErbB activity during androgen stimulated preantral follicle growth in vitro using receptor inhibitors.
- To compare ErbB expression in GL cells from control and PCOS women.

7.2 Results

7.2.1 DHT alters EGF-like ligand and ErbB receptor expression in preantral follicles in vitro

In order to investigate the hypothesis that androgens alter the sensitivity of follicles to EGF-like ligand signalling by regulating the expression of the ErbB receptors, preantral follicles were cultured with or without DHT and collected for mRNA analysis through quantitative RT-PCR. Follicles were collected at three time points: 6, 12 and 24 hours. DHT exposure had varying effects on ErbB receptor mRNA levels in cultured follicles. While after 6 hours in culture, DHT treatment significantly increased Egfr mRNA, there was a decrease in Erbb2 mRNA levels at 12 and 24 hours (Figure 7.2). Erbb3 mRNA levels were extremely low and occasionally undetectable in the samples, making the results highly variable and no significant difference between treatment was detected.
Figure 7.2 DHT increases *Egfr* but decreases *Erbb2* gene expression in cultured preantral follicles.
Preantral follicles cultured with vehicle control or DHT (10 nM) for 6, 12 or 24 hours. Gene expression data is displayed as fold change with DHT relative to control within each time point. Bars represent mean ± SEM (n = 6 ovaries, 6 follicles from same ovary pooled). Statistical analysis between control vs. DHT by unpaired t-test within time points, where *p < 0.05.

There remains no reliable antibody for immunofluorescence detection of EGFR protein, so unfortunately protein expression of EGFR with DHT treatment in preantral follicles could not be investigated. Western blotting may be used as an alternative method in future, as specific detection of EGFR protein was demonstrated in Chapter 3 in preantral follicles (see Figure 3.3), however the number of follicles that would need to be collected for sufficient protein lysate is substantial. DHT treated follicles were labelled for ERBB2 protein and showed a significant reduction in expression at 24 hours (Figure 7.3), mirroring the mRNA data for *Erbb2*. At 72 hours, ERBB2 protein was not significantly different between control and DHT treated follicles. ERBB3 was not investigated as the protein was shown to be absent from preantral follicles in Chapter 3 (see Figure 3.3).
Figure 7.3 DHT reduces ERBB2 protein expression in cultured preantral follicles. Preantral follicles cultured with DHT (10 nM) or vehicle control for 24 or 72 hours. A) Sections of follicles immunolabelled for ERBB2 (green) and counterstained with the nuclear stain DAPI (blue). Scale bar = 50 µm. B) Quantification of ERBB2 using ImageJ, calculated as percentage positive staining above a threshold in the GC compartment at 24 hours (n = 14 - 17 follicles) and 72 hours (n = 12 - 14 follicles). Bars represent mean ± SEM, statistically analysed by unpaired t-test within time points, where ** p < 0.01.

To investigate if androgens increase the expression and availability of EGF-like ligands, preantral follicles were cultured in vehicle control or DHT for 24 hours and collected for quantitative RT-PCR. The EGF-like ligands highlighted in Chapter 3 as the most highly expressed in preantral follicles were: Egf, Tgfa, Hbegf, Btc and Ereg. Transcript levels for Areg were too low to be reliably measured and Epgn was shown in Chapter 3 to be absent from preantral follicles (see Figure 3.7). DHT treatment caused a significant reduction in Tgfa and Btc mRNA levels at 24 hours, no difference was detected in Hbegf, Egf or Ereg between treatments (Figure 7.4). Availability of EGF-like growth factor ligands is regulated by their transcription as well as their proteolytic cleavage from their integral membrane precursors. Adam17 is a metalloprotease enzyme important in the processing of soluble ErbB ligands such as TGF-α and HB-EGF from their transmembrane precursors (Lee et al., 2003). Additionally, MMP14 has been implicated in the membrane shedding of HB-EGF (Overland and Insel, 2015). Levels of Adam17 mRNA were significantly decreased with DHT exposure, which could indicate a decrease in EGF-like ligand shedding in androgen exposed follicles. There was no difference detected in Mmp14 levels between treatment groups.
Figure 7.4 Effect of DHT on gene expression of EGF-like ligands in cultured preantral follicles.
Preantral follicles cultured with vehicle control or DHT (10 nM) for 24 hours. Gene expression data is displayed as fold change relative to control. Bars represent mean ± SEM (n = 5 ovaries, approx. 15 follicles from same ovary pooled). Statistical analysis between control vs DHT by unpaired t-test, where * p < 0.05, ** p < 0.01.

7.2.2 Combined EGF and DHT treatment has an additive effect on preantral follicle growth
Both EGF and DHT have been shown to significantly stimulate preantral follicle growth in culture when added individually (Figure 4.2 and Figure 6.2 respectively). In order to explore the presence of a synergistic relationship between the two ligands, medium was supplemented with a combination of EGF and DHT. It was hypothesised that as DHT had been shown to increase mRNA levels of Egfr which is the principal receptor relaying the EGF signal, increased EGFR expression may therefore augment the growth response of preantral follicles to exogenous EGF-like ligand. Exposure of preantral follicles to combined EGF and DHT resulted in a significant increase in size beyond that of individual treatments at 24 hours (Figure 7.5). At 48 and 72 hours however, combined treatment was only exaggerated significantly above that of the EGF treated follicles. The follicle area data revealed an additive effect of the two ligands, rather than a synergistic effect of follicle growth. Interestingly, visual comparison of the growth trajectories showed a difference in the response of follicles to EGF and DHT treatment. The response to EGF is rapid with the most pronounced effect at 24 hours before plateauing, while the
response to DHT is lagged but more sustained, displaying continuous increases at 48 and 72 hours.

**Figure 7.5** Combined DHT and EGF treatment stimulates preantral follicle growth above that of individual treatments in vitro.

A) Representative images of follicles cultured in vehicle control, EGF (10 ng/ml), DHT (10 nM) or combined EGF and DHT (10 ng/ml and 10 nM respectively), scale bar = 100 μm. B) Percentage change in follicle area relative to 0 hour is shown. Values are mean ± SEM, statistical analysis between treatments were made within each time point by one-way ANOVA and Tukey’s multiple comparison test, where * p < 0.05, ** p < 0.01. **** p < 0.0001 (n = 15 ovaries, 3 experiments). Stars above bars represent statistical difference from vehicle control. Numbers bracketed represent number of follicles in each treatment group.
Follicles treated with combined EGF and DHT were collected for mRNA extraction and analysed through quantitative RT-PCR for alterations in key gene expression. There was no significant change in mRNA levels of the ErbBs: Egfr, Erbb2 and Erbb3 (Figure 7.6). ErbB gene expression has been investigated previously with individual treatment of EGF (see Figure 4.13) or DHT (see Figure 7.2). Levels of Egfr mRNA were shown previously to be down regulated with EGF, and up regulated by DHT, suggesting the combination of treatments cancels out any overall effect of Egfr. Transcription of Erbb2 mRNA is repressed in both EGF and DHT treatments individually, interestingly however Erbb2 is not significantly decreased with combined treatment. Erbb3 mRNA is present at very low levels in isolated preantral follicles and does not differ between any treatment groups examined. Amh and Ar are both significantly down-regulated with DHT (see Figure 6.9) and EGF treatment individually (see Figures 4.13 and 4.14 respectively), however were unchanged in the combined treatment group. Fshr mRNA is significantly elevated in the presence of DHT (see Figure 6.9), but repressed with EGF (see Figure 4.13), the combined effect was not significantly different from controls.

Figure 7.6 Culture with combined EGF and DHT has no effect on Ar, Fshr, Amh or ErbB gene expression.
Preantral follicles cultured in vehicle control or EGF (10ng / ml) or DHT (10 nM) or combined EGF and DHT (10 ng/ml and 10 nM respectively) for 24 hours. Gene expression data is displayed as fold change from control. Bars represent mean ± SEM (n = 6 - 12 ovaries, 6 follicles from same ovary pooled). Statistical analysis between control vs treatment by unpaired t-test.
The increased preantral follicle size with combined treatment of EGF and DHT was investigated further through the immunolocalisation and quantification of proliferation marker Ki67 within GCs. It should be noted that data for individual treatment of EGF and DHT has been shown previously (see Figure 4.3 and Figure 6.4 respectively), but is included here for comparative purposes. Ki67 although elevated with individual treatment was not significantly changed from control with combined treatment at 24 hours (Figure 7.7A). This result was inconsistent with substantial increase in follicle size at this time point.

Both EGF and DHT individual treatments had dramatic effects on ERBB2 (EGF: see Figure 4.17, DHT: see Figure 7.3) and AR (EGF: see Figure 4.20, DHT: see Figure 6.11) protein expression in cultured preantral follicles. The differing growth trajectories promoted investigation into the effect of combined treatment on receptor protein expression. Again, it should be noted that data for individual treatment has been shown previously but is included for comparative purposes. Combined EGF and DHT augmented the down-regulation of ERBB2 protein expression at 72 hours (Figure 7.7B), repressing expression below that of EGF alone. The combination of EGF and DHT amplified the expression of AR protein significantly higher than individual EGF or DHT at 24 hours and above EGF at 72 hours (Figure 7.7C). Interestingly at 72 hours, AR expression in the combined treatment group was lower than DHT alone, although remained significantly elevated over vehicle control.
Figure 7.7 Quantification of Ki67, ERBB2 and AR protein in preantral follicles treated with combined EGF and DHT treatment.
Preantral follicles cultured with vehicle control, EGF (10 ng/ml) or DHT (10 nM) or a combination of EGF and DHT (10 ng/ml and 10 nM respectively) for 24 or 72 hours. Quantification of A) Ki67 (n = 12 - 17 follicles), B) ERBB2 (n = 10 - 18 follicles) and C) AR (n = 8 - 11 follicles) protein using ImageJ. Ki67 calculated as proportion of positively stained nuclei for Ki67, ERBB2 and AR calculated as percentage positive staining above a threshold in the GCs at 24 hours and 72 hours. Bars represent mean ± SEM, statistically analysed by one-way ANOVA with Tukey’s multiple comparison test within time points, where * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
7.2.3 DHT requires both EGFR and ErbB2 activity to elicit maximal stimulation on follicle growth

In order to investigate if the stimulatory effect of androgens on follicle growth was in part via modulation of EGF family signalling, various ErbB inhibitors were employed in combination with DHT treatment. Activated AR signalling may alter the production and shedding of EGF-like ligands, change the expression of ErbB receptors or activate the ErbB receptors through non-genomic mechanisms to relay a stimulatory signal.

To investigate if the EGFR subtype was involved in the mitogenic response seen with DHT on preantral follicle growth, the specific inhibitor AG1478, which selectively blocks the kinase activity of EGFR, was used to inhibit phosphorylation of the EGFR cytoplasmic tail. DHT stimulated follicle growth was significantly attenuated with the addition of AG1478 at 24 and 48 hours (Figure 7.8). The attenuation of DHT stimulated growth with AG1478 suggests EGFR activity is involved in AR action in preantral follicles. The inhibition of EGFR did not totally ablate the effects of DHT, as follicle growth with combined DHT and AG1478 remained significantly higher than control at 48 and 72 hours indicating that AR also acts through mechanisms independent of EGFR to stimulate preantral follicle growth.
Figure 7.8 EGFR inhibition reduces DHT stimulated preantral follicle growth in vitro.

Change in follicle area with AG1478 (10 µM) added in combination with DHT (10 nM). Values shown are mean ± SEM. Statistical analysis between treatments was performed within each time point by one-way ANOVA and Tukey’s multiple comparison test, where * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. Stars above bars represent statistical difference from vehicle control. Numbers in brackets are the number of follicles in each treatment group (n = 11 ovaries, 2 experiments).

The reduction in DHT stimulated follicle growth with AG1478 was investigated further and follicles were fixed at 72 hours. Follicles were labelled for either Ki67 or TUNEL and cleaved caspase-3 to identify proliferating and apoptotic cells. There was no significant change in Ki67 expression between follicles treated with DHT or combined DHT with AG1478 (Figure 7.9A), however the staining was highly variable. There was no significant change in TUNEL or cleaved caspase-3 with DHT or AG1478 (Figure 7.9B), suggesting the reduction seen in follicle growth was not caused by a toxic effect of the inhibitor.
Figure 7.9 AG1478 has no effect on DHT stimulated GC proliferation and does not induce apoptosis in preantral follicles in vitro.
Follicles cultured for 72 hours in vehicle control, DHT (10 nM) or DHT (10 nM) with AG1478 (10 µM) were fixed and stained for A) Ki67 (green) or B) TUNEL (green) and cleaved caspase-3 (red) and counterstained with nuclei label DAPI (blue), representative images are shown, scale bar = 50 µm. Protein was quantified as percentage positive staining above a threshold in the GC compartment in treated follicles at 72 hours. Ki67: Bars shown are mean ± SEM compared using unpaired t-test. TUNEL and cleaved caspase-3: Bars shown are medians, statistically analysed using Mann Whitney test.

The effect of ErbB2 inhibition on DHT stimulated follicle growth was investigated. At 48 and 72 hours, the addition of ErbB2 inhibitor ii significantly attenuated the stimulatory effect of DHT on growth (Figure 7.10), indicating that ErbB2 activity too is involved in mediating the follicular response to androgens. At all time points examined follicles exposed to DHT in combination with ErbB2 inhibitor grew significantly larger than with ErbB2 inhibitor alone, indicating that AR is likely also acting through mechanisms independent of the ErbB2 signalling pathway to stimulate preantral follicle growth.
**Figure 7.10 ErbB2 inhibition reduces DHT stimulated preantral follicle growth.**
Change in follicle area with ErbB2 inhibitor ii (40 µM) added in combination with DHT (10 nM). Values shown are mean ± SEM. Statistical analysis between treatments were made within each time point by one-way ANOVA and Tukey’s multiple comparison test, where * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \). Stars above bars represent statistical difference from vehicle control. Numbers in brackets are the number of follicles in each treatment group (\( n = 5 \) ovaries).

7.2.4 Inhibition of MAPK and PI3K signalling reduces DHT stimulated follicle growth

Androgen activation of the MAPK pathway has been reported in various other cell types (Sen et al., 2010, Mukherjee and Mayer, 2008, Hamzeh and Robaire, 2011, Peterziel et al., 1999, Zhu et al., 1999). Many of these studies have attributed the androgen-induced activation of the MAPK cascade to stimulation of ErbB signalling pathways. The MAPK pathway is known to be activated downstream of all EGFR and ErbB2 containing dimer combinations.

In order to investigate the involvement of MAPK signalling in DHT stimulated preantral follicle growth, the highly selective and potent inhibitor U0126 was employed. Via non-competitive inhibition U0126 inhibits the kinase activity of MEK1 and MEK2. The concentration of 10 µM was chosen based on dose response performed in Chapter 4 (Figure 4.8A) and to fit with previous literature published in the mouse ovary (Wang et al., 2013, Fan et al., 2004, Su et al., 2002). At 24 and 48 hours DHT stimulated follicle
growth was significantly reduced with U0126 (Figure 7.11). The inactive analogue U0124, included as a negative control, had no effect on baseline or DHT stimulated follicle growth. At 48 and 72 hours follicles exposed to DHT in combination with U0126 grew significantly larger than with U0126 alone, indicating that AR is also acting through MAPK pathway independent mechanisms to stimulate preantral follicle growth.

**Figure 7.11 MEK1/2 inhibition reduces DHT stimulated follicle growth.**

Percentage change in follicle area with DHT (10 nM) added in combination with either U0126 (10 µM) or its inactive analogue U0124 (10 µM) (n = 6 ovaries). Values shown are mean ± SEM. Statistical analysis between treatments were made within each time point by one-way ANOVA and Tukey’s multiple comparison test, where ns = non-significant, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. Stars and ns above bars represent statistical difference from vehicle control. Numbers in brackets are the number of follicles in each treatment group.

The PI3K pathway is another important signalling cascade activated downstream of the ErbBs. LY294002 hydrochloride is a complete and specific inhibitor of PI3K. There was a significant decrease in baseline follicle growth with LY294002 added alone at concentrations of 5 µM and 10 µM at all time points (Figure 7.12). Follicles cultured with the inhibitor alone displayed similar rates of survival to control, assessed by the number of follicles classed as morphological healthy at 72 hours. The restriction in baseline growth indicated that the PI3K/AKT pathway is essential for multi-layered preantral follicle growth. LY294002 treatment inhibited the stimulatory effect of DHT on preantral follicles growth with 5 µM at 24 hours and with 10 µM at all time points, and in the case of 10 µM to levels significantly below that of the vehicle control at 24
hours. Given the complete inhibition of growth in response to LY294002 alone it is unclear whether activation of the PI3K/AKT pathway is necessary for DHT stimulated follicle growth. However, at all time points follicles exposed to DHT in combination with 5 µM LY294002 grew significantly larger than with 5 µM LY294002 alone, indicating that AR stimulates preantral follicle growth through pathways independent of PI3K activity.

![Figure 7.12 PI3K inhibition reduces baseline and DHT stimulated follicle growth.](image)

**Figure 7.12 PI3K inhibition reduces baseline and DHT stimulated follicle growth.**
The effect of increasing concentrations of LY294002 (5 and 10 µM) alone and with DHT (10 nM) on preantral follicle growth in vitro (n = 5 ovaries). Values shown are mean ± SEM. Statistical analysis between treatments were made within each time point by one-way ANOVA and Tukey’s multiple comparison test, where * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. Stars above bars represent statistical difference from vehicle control. Numbers in brackets are the number of follicles in each treatment group.

### 7.2.5 Results using DHT conjugated to BSA to detect membrane signalling of AR are inconclusive

There is some evidence that androgens can signal from the cell membrane and interact with membrane bound receptors such as the ErbBs, however the molecular nature of membrane androgen binding sites remains unclear. One investigative method to uncover the presence of membrane steroid receptors is to use steroids conjugated to macromolecules, such as BSA that render the steroid impermeable to the cell membrane. This technique has been widely reported with oestrogen uncovering membrane ERs (Stevis et al., 1999) but little work has been documented with testosterone or DHT. DHT was purchased conjugated to BSA at the C3 position with
carboxymethyl-oxime (CMO) as the linker, creating DHT:CMO:BSA. DHT derivatives were dissolved at the molar equivalent concentration of DHT (10nM), and BSA was dissolved to achieve the molar equivalent concentration of BSA as in DHT:CMO:BSA. To ensure effects seen with DHT:CMO:BSA were not restricted due to chemical modification of the parent DHT molecule or altered affinity of DHT to AR, the control of DHT with linker bridge alone was included (DHT:CMO), which could traverse the cell membrane and activate AR with the same affinity as DHT alone.

DHT:CMO:BSA was unable to stimulate preantral follicle growth above the vehicle control at any time point (Figure 7.13), initially suggesting the lack of membrane androgen binding sites in preantral follicles. However follicle growth with DHT and the linker bridge alone (DHT:CMO) was significantly lower than DHT treatment at 48 and 72 hours, suggesting that the linking bridge alters the parent DHT binding affinity to AR. This additional control cast doubt over the initial conclusion, and although this result suggests the lack of membrane androgen binding sites, another protocol for investigating androgen membrane signalling should be considered to confirm the result.

Figure 7.13 Effect of DHT conjugated to BSA in preantral follicles in vitro.
The effect of DHT (10 nM), DHT:CMO (10 nM), DHT:CMO:BSA (10 nM) and BSA alone on preantral follicle growth. Values shown are mean ± SEM. Statistical analysis between treatments were made within each time point by one-way ANOVA and Tukey’s multiple comparison test, where ns = non-significant, * p < 0.05 **, p < 0.01, **** p < 0.0001. Stars and ns above bars represent statistical difference from vehicle control. Numbers in brackets are the number of follicles in each treatment group.
7.2.6 Human GL cells show no difference in ErbB expression between control and PCOS women

Variants in or close to three of the four ErbB genes (ERBB2/HER2, ERBB3/HER3 and ERBB4/HER4) have been linked to a PCOS diagnosis by a genome wide association study (Day et al., 2015), indicating a possible role for the ErbBs in the pathogenesis of PCOS. GL cells obtained during oocyte retrieval during routine IVF were collected and immediately taken for mRNA extraction and analysis through quantitative RT-PCR. No difference was detected in ErbB mRNA levels in women with PCOS compared to control (Figure 7.14).

![Figure 7.14 Human GL cells show no difference in ErbB mRNA levels between control and PCOS women.](image)

GL cells collected during oocyte retrieval during routine IVF were collected from control and PCOS women. mRNA was immediately extracted and quantitative RT-PCR run to detect; EGFR, ERBB2/HER2, ERBB3/HER3 and ERBB4/HER4. Values shown are mean ± SEM, n = 10 control samples, n = 11 PCOS samples.

7.3 Discussion

7.3.1 Androgens regulate EGF family expression in preantral follicles

The first hypothesis examining crosstalk between androgens and the EGF family signalling pathway, investigated the effect of androgens on ErbB receptor expression in preantral follicles. Interestingly, exposure to DHT had varying effects on the ErbB receptors in cultured preantral follicles, increasing Egfr mRNA levels but repressing ErbB2 mRNA and protein expression.

The ErbB subtype EGFR was highlighted in Chapter 3 as the principal receptor through which the mitogenic response to EGF ligand was transmitted. Therefore, a DHT stimulated increase in EGFR expression may increase the sensitivity of follicles to
signalling from endogenous EGF-like ligands produced within the follicle (identified in Chapter 3). This increased sensitivity to growth factor ligands may provide a mechanism contributing to the stimulatory effects of androgens on follicle development. Similarly, DHT has been shown previously to regulate expression of other growth factor receptors, such as the IGF1R, altering sensitivity to IGF ligands and indirectly stimulating follicle progression. Recent in vitro work produced within our group has demonstrated that neonatal mouse ovaries cultured in the presence of DHT display a significant increase in IGFR1 protein expression. The increase in IGF1R expression resulted in elevations in downstream cytoplasmic Foxo3 expression (unpublished, Mhairi Laird). Active Foxo3a resides in the nucleus and functions at the earliest stages of follicular growth as a suppressor of follicular activation, but DHT stimulated movement of Foxo3a to the cytoplasm releases the inhibitory signal and induces follicular development (Castrillon et al., 2003). Furthermore, in vivo evidence from work performed in the Rhesus monkey has shown that androgen stimulated follicle growth is attributable to an androgen mediated increase in the expression of IGF1R in granulosa, thecal and interstitial cells (Vendola et al., 1999).

Androgen enhancement of EGFR expression has been reported in other cell types including the human endometrium (Watson et al., 1998). Endometrial cells incubated with either testosterone or DHT displayed an increase in EGFR concentration (96% and 144% higher than control respectively) but not receptor affinity, causing an increase in [125I]-labelled EGF specific binding. The stimulatory effect of testosterone was inhibited by the anti-androgen hydroxyflutamide, but not by the anti-oestrogen ICI182780 or the aromatase inhibitor 4-hydroxyandrostenedione, proving the results were caused by an androgenic effect. The authors postulated that the irregularities in endometrium thickness in women with PCOS may be linked to androgen mediated modulation of EGF signalling (Watson et al., 1998). An androgenic mediated increase in growth factor sensitivity in preantral follicles, may contribute to the understanding of the abnormalities seen in polycystic ovary. A high androgenic environment around the developing follicle may result in increased sensitivity to growth factor ligands stimulating preantral follicle development. Ovarian cortical biopsies taken from patients presenting with polycystic ovaries display a significantly higher proportion of early
growing follicles (Webber et al., 2003), this has been linked to androgen excess but the underlying mechanisms remain unknown.

Multiple studies have reported androgen mediated increases in EGFR mRNA and protein expression in androgen sensitive cancer cells, such as bladder (Zheng et al., 2011) and prostate (Brass et al., 1995). The mechanisms by which androgens stimulate the expression of EGFR have been investigated in these cells. Studies in prostate cancer LNCaP cells have shown that DHT can stimulate RNA polymerase II recruitment to the EGFR gene promoter to stimulate EGFR mRNA transcription and increase EGFR protein levels (Pignon et al., 2009). In another prostate cancer cell line, androgen ablation-resistant 22Rv1 cells, DHT altered protein expression of EGFR through posttranscriptional regulation (Pignon et al., 2009). However, it is important to note in the present study that enhanced EGFR was only seen at the transcriptional levels and investigation into EGFR protein expression following androgen treatment needs to be examined.

Interestingly, there was a decrease in Erbb2 mRNA and ERBB2 protein expression in DHT treated follicles. As ErbB2 expression is often associated with increased proliferation, implicated in the progression of many cancers, this result was surprising. The opposing dual regulation of EGFR and ErbB2 subtypes has been reported in other cell types. Several studies in LNCaP prostate cancer cells have reported that DHT treatment increased EGFR while simultaneously decreased ErbB2 transcript and protein expression (Pignon et al., 2009, Myers et al., 1996).

The decrease in both ErbB2 mRNA and protein expression may indicate that AR acts at the gene promoter to repress Erbb2 transcription. However, it remains undetermined if this is through direct AR binding or through indirect mechanisms. The dramatic drop in ERBB2 protein expression at 24 hours could also indicate an androgen stimulated increase in receptor internalisation and degradation. Most literature to date has focused upon EGFR ligand-activated endocytosis, although activation of ErbB2 also results in receptor internalisation (Sorkin and Goh, 2009, Roepstorff et al., 2008). However, ErbB2 receptors are internalised less efficiently than EGFR, considered to be endocytosis impaired, and instead of being targeted for lysosomal degradation, ErbB2 dimers are
largely recycled back to the plasma membrane for reactivation. Therefore, the effects seen with DHT on protein are likely mediated via a decrease in \textit{Erbb2} transcription.

The principal role for ErbB2 in the developing follicle is unclear. Although ErbB2 expression contributes to relaying the mitogenic response to EGF, the immunolocalisation of ErbB2 protein to cuboidalising GCs, suggests additional roles for this protein. The remarkably similar protein localisation pattern of ErbB2 and N-cadherin (Mora et al., 2012) led to the suggestion in Chapter 3 that ErbB2 may regulate the assembly of N-cadherin based adherens junctions, playing a role in GC cuboidalisation and the partial EMT following activation. The significant decrease in ErbB2 expression with DHT may have other effects on follicular development besides proliferation, in particular cell adhesion and cuboidalisation, and should be investigated further.

The decrease in ErbB2 may lead to altered ErbB dimer combinations formed upon ligand stimulation. ErbB2 containing heterodimers are proposed to be the most potent complexes (Pinkas-Kramarski et al., 1996), as ErbB2 can potentiate and prolong signal transduction pathways, enhance ligand affinity and relax ligand specificity. Additionally the presence of ErbB2 can alter receptor trafficking, as ErbB2 containing heterodimers are preferentially recycled back from endosomes, and can prolong ErbB expression at the cell membrane (GrausPorta et al., 1997). Androgen mediated alterations in relative ErbB expression may alter downstream signalling outcomes from EGF-like ligand signalling within the developing follicle.

Exposure of follicles to prototypical EGF significantly increased follicle growth \textit{in vitro}, shown in Chapter 4, therefore the hypothesis was presented that DHT may be regulating the availability of stimulatory EGF-like ligands to increase follicle growth. However, DHT treatment significantly repressed both \textit{Tgfa} and \textit{Btc} mRNA levels, demonstrating that DHT does not increase EGF-like ligand transcription. However, DHT has been shown to stimulate the MMP-mediated shedding of membrane bound EGFR ligands, such as HB-EGF, in other cell types. It has been shown by others that medium collected from prostate cancer LnCAP cells cultured with DHT increased EGFR phosphorylation when added to A431 cells, while medium from vehicle treated cells did
not (Sen et al., 2010), indicating that DHT stimulated the shedding of EGFR ligands into the culture medium. The authors also showed that the addition of galardin, a MMP inhibitor, blocked this effect, demonstrating the shedding was dependent on MMP activity. In the present study, DHT decreased Adam17 mRNA levels and left Mmp14 levels unchanged. However activity of these cleavage enzymes was not assessed and it is possible that DHT stimulates the activity rather than expression of these proteins. Future studies could investigate the shedding and availability of EGF-like ligands into the culture medium following DHT treatment. Also protein expression of the EGF-like ligands should be investigated.

7.3.2 Combined EGF and DHT has an additive stimulatory effect on preantral follicle development

Preantral follicle cultures exposed to combined EGF and DHT were used to investigate a synergistic effect of the two signalling pathways on follicle growth. The novel combination of EGF and DHT supplementation significantly increased growth above that of the individual treatment demonstrating an additive effect on follicle growth. There was no evidence for a synergy between the two ligands, i.e. the interaction of treatments when combined did not produce a total effect that is greater than the sum of the individual treatments. Interestingly, combined cultures revealed that the growth trajectories in the response to separate EGF or DHT stimulation were different. This is likely linked to receptor expression post-treatment. EGF treatment down-regulates expression of its own receptors (EGFR and ErbB2) at both the mRNA and protein level (see Chapter 4), and has been shown by other studies in induce ligand-mediated receptor endocytosis, which likely mutes the response to EGF in the latter period of the follicle culture. In contrast, DHT treatment enhances AR protein expression in follicles over culture (see Chapter 6) sensitising them further to androgen exposure.

Earlier studies have provided evidence for a synergy between the two ligands in other cell types, based on two hypotheses. First, that androgen treatment can stimulate an increase in ErbB expression or activity, and second that EGF signalling can increase expression or augment activation of AR.
The former presented a possibility as DHT treatment increased *Egfr* mRNA in cultured preantral follicles, potentially sensitising the preantral follicle to EGF signalling. Evidence for a similar synergy comes from work performed in PC3 prostate cancer cells. In cultured PC3 cells, DHT treatment caused a two-fold increase in *EGFR* mRNA and a 50% rise in EGFR protein compared to control which caused elevated EGF binding (Brass et al., 1995). The increase in EGFR expression resulted in a synergistic effect on cell growth when EGF and DHT were added in combination to culture.

Additionally, EGF treatment was shown to increase AR protein expression in cultured preantral follicles in Chapter 4, which may increase sensitivity to androgens. EGF signalling has also been shown to augment the effects of androgen in some carcinogenic tissues. EGF signalling has been shown to activate AR in the absence of sufficient androgen ligand in prostate cancer cells, encouraging cell proliferation and migration (Guo et al., 2006). Also in prostate cancer cells, dual inhibition of EGFR/ErbB2 reduces AR transcriptional activity, and signalling through ErbB2 in particular has been shown to stabilise AR protein levels and optimise binding of AR to promoter regions of androgen-regulated genes (Mellinghoff et al., 2004). The enhancement of AR activation may be mediated in part by increased expression or phosphorylation of AR or nuclear receptor co-activators such as TIF2/GRIP1 (Gregory et al., 2004). It should be noted that almost all studies concerning EGF augmentation of AR activation have been performed in carcinogenic tissue and the relevance to healthy tissue is unknown.

Along with the additive effect of EGF and DHT on follicle growth, immunohistochemical analysis revealed evidence for an augmentation above the individual effects on ERBB2 and AR protein expression. Surprisingly though, staining for Ki67 expression in the combined group was significantly lower than EGF and DHT treatment individually, indicating lower GC proliferation. This decrease in Ki67 staining with combined EGF and DHT treatment was unexpected and inconsistent with the increase in follicle size at this time point. The lack of proliferation may suggest a decrease in GC apoptosis with the combined treatment, which could be investigated further with staining for apoptosis markers such as TUNEL and cleaved caspase-3.
7.3.3 DHT stimulated follicle growth involves activation of the ErbBs

The addition of various ErbB inhibitors of EGFR and ErbB2 significantly attenuated DHT stimulated preantral follicle growth. This reduction suggests that ErbB activity is required, in part, for DHT stimulated follicle development. There are several potential hypotheses for this finding (Figure 7.15).

**Figure 7.15 Proposed mechanisms of AR - EGF family crosstalk.**

DHT within the cell cytoplasm binds to and activates AR. AR signals either within the cytoplasm (dotted grey line) or translocates to the nucleus to regulate gene transcription (complete grey line). ErbB activity is required for maximal androgen stimulation of preantral follicle growth. Androgens may crosstalk with the EGF family to increase proliferation through the following hypothesised mechanisms: 1) Androgens increase ErbB expression in the cell membrane, through increased transcription or altered recycling 2) Androgens increase expression or cleavage of EGF-like ligands from their membrane bound precursors or 3) Androgens signal through non-genomic pathways to activate the ErbBs. Turquoise receptor represents ErbB dimerisation partner. HSP, heat shock protein; AR, androgen receptor; DHT, dihydrotestosterone; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase.

Firstly, as previously discussed, DHT actions may be mediated partly through increased sensitivity to endogenous EGF-like ligands by increased ErbB expression at the cell surface (Figure 7.15.1). DHT has been shown to increase levels of *Egfr* mRNA.
Additionally, DHT treatment may alter trafficking of the ErbB receptors. Upon activation, ErbB dimers are internalised and sorted by early endosomes. Androgens may alter ErbB trafficking fates, causing ErbB dimers to be preferentially recycling back to the cell surface rather than targeted for degradation by lysosomes, however this hypothesis requires further investigation. EGF-like ligands are known to be expressed within the preantral follicle therefore removal of this endogenous signalling may dampen the effects of DHT on follicle growth.

Secondly, DHT may alter the availability of EGF-like ligands (Figure 7.15.2). The quantitative RT-PCR screen in this chapter demonstrated that DHT did not increase the transcription of EGF-like ligands in culture. However, it should not be excluded that DHT may stimulate the protein expression or shedding of membrane bound ligands, as shown in other cell types. An increase in the availability of EGF-like ligands would contribute to the stimulatory effect on preantral follicle growth through extra-cellular activation of the ErbBs.

Thirdly, AR may signal through activation of the ErbBs, through non-genomic signalling pathways to promote proliferation and differentiation (Figure 7.15.3). AR mediated rapid activation of EGFR has been shown in other model systems, either through intermediate kinases such as Src within the cytoplasm (Cheng et al., 2007a, Migliaccio et al., 2007), or through the rapid MMP-mediated shedding of EGF-like ligands from the membrane, which activate EGFR (Sen et al., 2010). Although AR non-genomic signalling has not yet been shown in mouse preantral follicles, one study using cultured mouse GCs has shown that rapid MAPK phosphorylation with DHT is ablated with the addition of EGFR inhibitor AG1478 as well as the MMP inhibitor, Galardin. This study indicates that androgens stimulate MMP-mediated shedding of membrane bound EGFR ligands to elicit downstream MAPK signalling in mouse GCs (Sen et al., 2014). There is limited evidence for AR mediated ErbB2 activation. However, membrane ERα receptors have been proposed to interact and trans-activate ErbB2 containing heterodimers in the membrane, with subsequent activation of the PI3K-Akt pathway (Stoica et al., 2003). Evidence for non-genomic signalling in developing follicles is explored further in Chapter 8.
It has been suggested that non-genomic AR signalling through EGFR promotes and enhances intra-nuclear genomic effects (Hammes and Davis, 2015, Sen et al., 2012). In prostate cancer cells it has been proposed that maximal AR transcription in the nucleus requires extra-nuclear AR signalling (Hammes and Levin, 2007). Although the mechanisms remain unclear, in prostate cancer cells, non-genomic MAPK signalling via EGFR phosphorylates an intermediate molecule called paxillin (Sen et al., 2010), a cytoplasmic adaptor protein, which translocates to the nucleus and can bind to androgen stimulated AR. The paxillin AR complex promotes binding to gene promoters to regulate transcription, and can retain AR within the nucleus to prolong signalling (Sen et al., 2012). The authors proposed that paxillin acts as a liaison between extranuclear MAPK signalling and nuclear AR transcription. The removal of the ‘liaison’ phosphorylated paxillin using paxillin-specific siRNAs significantly reduces prostate cancer growth, migration and invasion (Sen et al., 2010). A recent study by Sen et al. reported that DHT stimulated survival of isolated mouse follicles through an increase in antiapoptotic miR-125b expression (Sen et al., 2014). Although this increase was mediated via genomic AR binding to AREs situated in the promoter region of the miR-125b-2 gene (shown through ChIP assay) the elevation was lost with the addition of MEK inhibitor U0126 or siRNA-mediated knockdown of paxillin. The authors proposed that the androgen-triggered extranuclear MAPK3/1 signalling was essential for nuclear AR-mediated transcription of miR-125b in mouse GCs.

In all cases follicles subjected to EGFR and ErbB2 inhibition in combination with DHT still grew significantly above control. These results indicate that AR also signals independently of the ErbBs. This was expected, as AR is known to signal primarily as a ligand activated transcription factor, moving directly to the nucleus following stimulation to regulate gene transcription.

### 7.3.4 DHT stimulated follicle growth may involve MAPK and PI3K signalling

The inhibition of signalling cascades known to be activated downstream of the ErbBs restricted DHT stimulated follicle growth. The MEK1/2 inhibitor U0126 attenuated DHT stimulated follicle growth, indicating that the MAPK signalling cascade plays a role in DHT actions on follicle development.
Androgen activation of the MAPK pathways has been shown in various other tissues (Sen et al., 2010, Mukherjee and Mayer, 2008, Hamzeh and Robaire, 2011, Peterziel et al., 1999, Zhu et al., 1999). A recent study published by Sen et al., has demonstrated that androgens can promote follicle development independent of transcription through the MAPK pathway (Sen et al., 2014). DHT added to mouse primary GC cultures resulted in increased FSHR protein but not Fshr mRNA. There was no reported change in protein degradation with DHT, therefore the authors speculated that DHT promotes FSHR protein expression by enhancing translation through extra-nuclear AR signalling. Moreover, the addition of the MEK inhibitor U0126 to culture reduced DHT stimulation of FSHR protein implicating the MAPK in this extra-nuclear signalling. Our work disagrees with this study in that DHT was found to positively regulate Fshr mRNA in cultured preantral follicles and isolated GCs, however it may suggest that DHT acts to promote FSHR expression through both transcriptional and post-transcriptional processes.

However, it should be noted that the inhibitors alone appeared to inhibit follicle growth, significantly with LY294002. This indicates that these signalling pathways, particularly the PI3K pathway, are essential for multi-layered preantral follicle growth. Many regulators of preantral follicle size produced within the follicle are known to converge on the MAPK and PI3K signalling cascades. By knocking out these signalling pathways, there is likely indirect effects of other endogenous ligand signalling within the follicle such as KL (John et al., 2009). It therefore cannot be categorically concluded that DHT is signalling through these pathways, as their removal impacts on signalling from other ligands and restricts the ability of the follicle to grow normally. However, it equally cannot be excluded that DHT may stimulate the MAPK and PI3K/AKT signalling pathway to produce a mitogenic effect. Future experiments could be repeated with a lower dose of LY294002 that does not completely inhibit baseline follicle growth.

7.3.5 Membrane AR binding sites require further investigation

The proposed mechanisms of non-genomic androgen signalling are controversial. Some authors have proposed that AR may reside and signal within the cell membrane to elicit a non-genomic response. About 5% of oestrogen receptors (ERα and ERβ) are proposed to be membrane bound (Hammes and Davis, 2015, Pedram et al., 2007, Razandi et al.,
and several studies have localised AR at the cell membrane (Freeman et al., 2005, Pelekanou et al., 2010), one study in the ovary (Braun and Thomas, 2004). One study has shown that AR interacts directly with the integral membrane protein caveolin-1, suggesting that AR exists in plasma membrane structures primarily in lipid rafts within the membrane (Freeman et al., 2005). The presence of membrane AR binding sites was investigated in developing preantral follicles with membrane impermeable DHT conjugated to BSA. This experimental method has previously been used successfully and published in the literature (Somjen et al., 2009, Somjen et al., 2004, Gatson et al., 2006, Gatson and Singh, 2007). In the study described here, DHT conjugated to BSA was unable to elicit a stimulatory effect on preantral follicle growth, indicating the absence of membrane AR binding sites. Unfortunately, the linking bridge alone (DHT-CMO) blocked DHT stimulated follicle growth, most likely obstructing successful binding to AR. The results were therefore difficult to interpret and no conclusion could be drawn regarding the presence of AR in the cell membrane from this study.

7.3.6 ErbB expression is similar between control and PCOS women

Despite PCOS being the most commonly diagnosed reproductive disorder in women, the aetiology remains unclear. A recent genome wide association study found variants in or close to three of the four ErbB genes (ERBB2/HER2, ERBB3/HER3 and ERBB4/HER4) at or near genome-wide significance linked with a PCOS diagnosis. The strongest PCOS signal with an odds ratio of 1.18 was an intronic variant in the ERBB4/HER4 gene (Day et al., 2015). The authors suggest a role for the ErbBs in the pathogenesis of PCOS.

Earlier findings demonstrated with mouse preantral follicles that androgens play a role in the regulation of the ErbB receptor expression in the ovary. As women with PCOS commonly present with hyperandrogenism, it was hypothesised that the abnormalities in androgen production in women with PCOS may alter the transcription and expression of the ErbB receptors in the human ovary. However, there was no difference detected in ErbB mRNA levels in GL cells taken from women with PCOS compared to control. This may suggest that the variants found in the genome wide association study do not translate to altered transcription of the ErbBs in the ovary, and may instead alter protein expression, signalling capabilities or recycling of the ErbB receptors.
7.4 Conclusions

Androgen treatment alters both the expression of ErbB receptors and EGF-like ligands in preantral follicles in vitro, possibly altering the sensitivity of preantral follicles to growth factor signalling. The novel combination of DHT and EGF treatment elicited an additive effect of the two ligands on preantral follicle growth in culture. Maximal effects of DHT on preantral follicle growth require activity of the ErbBs, however the mechanisms by which androgens stimulate ErbB signalling are still unclear.
Chapter 8 Non-genomic androgen signalling in the ovary

8.1 Introduction

Androgens exert their effects primarily through AR, the ligand-activated nuclear transcription factor. AR signals classically through genomic pathways to regulate gene transcription in the nucleus (Mangelsdorf et al., 1995). However, it is becoming clear that AR can also exert effects beyond the nucleus to induce rapid, non-genomic actions (Foradori et al., 2008). These non-classical mechanisms of androgen signalling often involve crosstalk with either G-proteins and kinases or growth factor receptors. One of the most well documented mechanisms of androgen-growth factor crosstalk during non-genomic signalling involves the ErbBs, with evidence widely reported in other cell types such as Sertoli cells and prostate cancer cells (Sen et al., 2011). There has been little research into the presence of non-genomic signalling in developing follicles; however, there is emerging evidence that the effects of androgens in some ovarian target tissues depend upon extranuclear signalling pathways. Neonatal mouse ovaries exposed to testosterone display activation of the PI3K/AKT pathway within minutes of exposure (Yang et al., 2010). Additionally, non-genomic testosterone signalling has also been shown to induce mammalian oocyte maturation, independent of transcription involving the activation of MAPK and CDK1 signalling in mouse oocytes (Gill et al., 2004).

Classic genomic signalling of AR can take hours to yield a response, while AR non-genomic signalling can elicit phosphorylation events within seconds or minutes post-stimulation. Investigation into whether DHT could stimulate rapid phosphorylation of second messenger proteins in developing follicles was therefore undertaken. The preantral follicle culture system proved impractical for the examination of rapid phosphorylation events. Impracticalities arose due to the lengthy process of moving individual follicles, making short time points challenging, as well as the limited protein available from follicles for analytical western blotting. Therefore, a primary monolayer culture system was established using GCs from antral follicles collected from the d26 mouse ovary. The optimisation of the mouse GC culture system is included in this
chapter. Cells could be easily exposed to androgen stimulation for short periods and there was sufficient protein collected so that activation of downstream phosphorylation events within the MAPK and PI3K signalling cascades could be investigated through western blotting.

Additionally, human GL cells collected from women undergoing routine IVF treatment were taken for culture and treated with DHT for short time points to investigate the presence of rapid non-genomic androgen signalling in the human ovary.

**8.1.1 Specific aims**

- To establish and optimise a mouse GC monolayer culture system.
- To investigate the presence of non-genomic rapid signalling in mouse GCs, and uncover the mechanisms underlying this signalling.
- To investigate the presence of non-genomic androgen signalling in the human ovary.

**8.2 Results**

**8.2.1 Optimisation of GC monolayer culture system**

In order to detect rapid second messenger phosphorylation in developing follicles a GC monolayer culture was developed and optimised. GCs were extracted from d26 mouse ovaries that consisted of primarily preantral and small antral follicles. GC extraction was undertaken using acupuncture needles to puncture follicles followed by the collection of extruded GCs. The limited abundance of GCs meant that cells extracted from two mice (four ovaries) had to be pooled for each biological repeat to yield sufficient protein. The process was highly labour intensive and required two people working in parallel, therefore all GC cultures included in this chapter were established with the generous assistance of Jocelyn Mora.

The initial objective was to obtain a pure GC population. During the isolation process extruded oocytes from punctured follicles were inevitably collected along with GCs. Visual clearance of oocytes was performed under a dissection microscope, however many remained in the collected cell population. In order to remove contaminating
Chapter 8: Non-genomic androgen signalling in the ovary

Oocytes, the mixed cell population was passed through filters of differing pore size (Figure 8.1). Filters with 70 µm pores were ineffective, while filters with smaller 40 µm pores successfully excluded all contaminating oocytes. However, there was substantial cell loss during the filtration process. As well as oocytes, large clumps of GCs remained trapped in the filter. This concern is addressed and rectified later (see Figure 8.4).

![70µm Filter and 40µm Filter with magnifications](image)

**Figure 8.1 40 µm filter successfully removed oocytes during GC isolation.**
GCs and oocytes were extracted from d26 ovaries and passed through filters of differing pore size (40 µm and 70 µm). Representative images of isolated cells are shown post-filtering at x 10 and x 20 magnification. Scale bar = 200 µm.

Various culture conditions were explored. Cells were filtered with a 40 µm pore size filter, and grown with and without attachment factor (a gelatin containing solution which coats the well of the culture plate to promote attachment of cells), in increasing concentrations of FBS (2% and 10%). Following 72 hours in culture, morphology and confluence of cells was assessed visually (Figure 8.2A). Additionally, cell proliferation was analysed quantitatively through the measurement of protein concentration per well determined by a BCA protein assay kit (Figure 8.2B). Presence of an attachment factor made no difference to morphology or protein content and was therefore not necessary or included in future cultures. GCs grown in both 2% and 10% FBS were prolific and appeared morphologically healthy. Cells exposed to 10% FBS were more confluent at 72
hours and yielded higher protein content. However, the high FBS content caused problems with spontaneous luteinisation, addressed in the following figure (see Figure 8.3B).

Figure 8.2 GCs appeared healthy and more confluent with 10% FBS with no AF. A) GCs were grown for 72 hours with or without attachment factor (AF) in 2% or 10% FBS. Representative images taken at 24 hours are shown, Scale bar = 200 µm. B) Protein content per well was assessed using a BCA protein assay kit, concentration of protein lysate and total protein per well is shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of protein (µg / mL)</th>
<th>Total protein /per well (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% FBS Attachment Factor</td>
<td>323.4</td>
<td>25.9</td>
</tr>
<tr>
<td>10% FBS Attachment Factor</td>
<td>475.0</td>
<td>38.0</td>
</tr>
<tr>
<td>2% FBS No Attachment Factor</td>
<td>418.2</td>
<td>33.4</td>
</tr>
<tr>
<td>10% FBS No Attachment Factor</td>
<td>513.5</td>
<td>41.1</td>
</tr>
</tbody>
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GCs were maintained for 7 days in culture and photographed every 24 hours. Cells appeared morphologically healthy for up to a week in culture (Figure 8.3A). Cells were collected at 24 hour intervals and taken for mRNA extraction and gene analysis through quantitative RT-PCR. Genes associated with a granulosa phenotype were examined as well as genes characteristic of luteinised cells. Classic granulosa markers, Amh and Fshr, decreased dramatically over the culture period, and were significantly lower at 1 week compared to 24 hours (Figure 8.3B). There was a simultaneous increase in the
steroidogenic marker of luteinisation, Star. These results strongly suggested that GCs were spontaneously luteinising over the culture period. Interestingly, there was also an increase in Ar mRNA levels over culture. Data shown in Figures 8.3 and 8.5-8.7 are courtesy of Harriet Folland (HF, BSc project, 2015). Experiments were conceived and designed by Kacie Thomson (KT) and Jocelyn Mora (JM). Ovaries were collected and cells were isolated, cultured, treated and collected by KT and JM, while photography, quantitative RT-PCR and immunohistochemistry was performed by HF under supervision from KT and JM.

Figure 8.3 GCs showed signs of spontaneous luteinisation over culture period.
A) Representative images of GCs cultured over a week and photographed every 24 hours, scale bar = 100 µm. B) Gene expression data is displayed as fold change from 24 hours. Bars represent mean ± SEM (n = 3 biological repeats, pooled GCs from 2 mice / 4 ovaries / repeat). Statistical analysis between time-points by one-way ANOVA and Tukey’s multiple comparison tests, where * p < 0.05, ** p < 0.01. Data courtesy of HF, BSc project, in collaboration with KT and JM.
Chapter 8: Non-genomic androgen signalling in the ovary

The spontaneous differentiation and luteinisation of GCs over the culture period was concerning, as the results obtained in later studies may not be truly characteristic of GCs from the developing follicle. The principal determinant controlling luteinisation appeared to be time in culture, but concentration of FBS has also been shown by others to play a role. In early studies examining human co-cultures of theca and GCs, cells cultured in serum-supplemented medium resulted in increased progesterone production, a feature of luteal cells, relative to cells cultured alone (Batta et al., 1980). Culture conditions optimised to prevent spontaneous luteinisation have been explored in other model species. In bovine GCs basal and FSH-induced oestrogen production, a characteristic of granulosa like cells, could be maintained long-term in serum free conditions while cells cultured in serum -coated culture wells displayed relatively lower oestradiol production (Gutierrez et al., 1997a). The bovine GCs cultured in serum free conditions maintained morphological characteristics of GCs; spherical and tightly joined by extensive gap junctions and interdigitated pseudopodia/microvilli, had abundant rough and smooth ER and mitochondria with trabecular cristae, whereas cells cultured in serum-coated wells possessed morphological characteristics suggestive of early luteinisation; enlarged, containing less rough ER, had fewer mitochondria and contained endosome-like structures (Gutierrez et al., 1997b). The composition of FBS is undefined and may include growth factors, cytokines and possibly gonadotropins. Although LH is the main trigger for luteinisation, growth factors such as IGF-1 can also hasten luteinisation in vitro, shown to stimulate the appearance of StAR during in vitro luteinisation in porcine GCs (Pescador et al., 1999).

However, others have reported that supplementation of medium with serum is not essential for in vitro luteinisation, and may in fact attenuate hormone secretion. Luck reported that bovine GCs cultured in collagen-pretreated wells began to spontaneously secrete oxytocin and progesterone from their first few days in culture in serum-free conditions (Luck, 1989, Luck et al., 1990). Furthermore, the addition of serum to culture medium reduced oxytocin secretions by up to 90%. The authors found that oxytocin and progesterone concentrations showed a positive exponential relationship with cell density over the culture period. Therefore, they hypothesised that the ability of serum to promote spontaneous luteinisation may be a subsequent effect of increased cell attachment which allows for quicker propagation of the cells.
The removal of oocyte signalling may also accelerate GC luteinisation \textit{in vitro}. Early studies had shown that GCs from mature follicles obtained from oestrous rabbits when either punctured or oocytectomised, displaying histological signs of luteinisation after 3 days (el-Fouly et al., 1970). Additionally, mouse oocytes have been shown to regulate GC steroidogenic pathways \textit{in vitro}, as murine oocyte-GC complexes from preantral and antral follicles when oocytectomised secrete higher levels of progesterone than intact complexes. Furthermore, progesterone accumulation by oocytectomised complexes was inhibited when complexes were co-cultured with oocytes (Vanderhyden and Macdonald, 1998). These studies indicate that the oocyte maintains the granulosa phenotype and supports oestrogen synthesis from the GCs.

In order to encourage the maintenance of the GC phenotype and prevent spontaneous luteinisation over culture several steps were taken:

1. Cells were not filtered before plating. The removal of the filtering process meant that extruded oocytes were not removed and were plated alongside GCs. The inclusion of oocytes meant that oocyte-GC signalling networks were maintained, likely encouraging preservation of the GC phenotype. It was observed that oocytes did not bed down during the culture period, meaning they were successfully removed during the PBS washes proceeding protein extraction. The resultant protein lysate therefore contained very little if any oocyte protein contamination. This had the additional benefit of reducing the substantial cell loss during the filtration process, increasing GC cell counts.

2. Cells were maintained for a maximum of 48 hours before treatment. The changes indicative of luteinisation seen in gene expression were less pronounced earlier in the culture (Figure 8.3B) therefore for all further experiments cells were collected at or before 48 hours. The removal of the filtering process meant that cell counts were dramatically increased. Additionally, the cells clumped together in large groups, which had been previously removed by the filtering process, were now plated and successfully spread out over the culture period to form a monolayer culture. These cell masses contained proportionately less apoptotic cells during the hemocytometer counts than individual GCs, measured by trypan blue staining.
increasing average cell viability. The increase in initial cell numbers along with increased viability resulted in cells reaching confluence earlier in culture.

3. Serum concentration was dropped from 10% to 2% FBS for the first 24 hours, then 0% until treatment. Although previous work (Figure 8.2) had shown an increase in GC proliferation with higher serum concentrations, lower concentrations were sufficient to maintain cell viability and were more likely to prevent luteinisation. Cells were plated in 2% FBS and allowed to attach overnight, cells were then maintained in serum free medium for the subsequent 24 hours before treatment.

These three changes to the GC culture protocol increased GC viability, increased protein yield and successfully maintained GC morphology (Figure 8.4). Another quantitative RT-PCR experiment should be repeated investigating granulosa and luteal cell markers, to confirm the maintenance of granulosa identity over culture, however due to time limitations of this project this is yet to be completed.

**Figure 8.4 GC optimised culture conditions.**
Representative images of GCs during culture with the optimised protocol. Photos show oocytes in culture which did not attach, and show cells reached full confluence after 48 hours. Scale bar = 200 µm.

**8.2.2 Cultured GCs express AR protein and are DHT responsive**
With establishment of the GC culture conditions, it was then investigated if cultured GCs expressed AR protein and were therefore capable of responding to androgen signalling. Cells were cultured on coverslips for 48 hours and fixed in 4% paraformaldehyde. Cells were probed for AR protein using immunofluorescence staining (Figure 8.5). GCs expressed AR protein following 48 hours in culture, protein was localised around and
within the cell nucleus. Immunohistochemistry was performed by HF (BSc project, 2015) under supervision from KT and JM.

![Figure 8.5 Untreated GCs express AR protein following 48 hours in culture.](image)

**Figure 8.5 Untreated GCs express AR protein following 48 hours in culture.**

GCs cultured for 48 hours and immunostained for AR (red) and counterstained with nuclear label DAPI (blue). A) Representative image of multiple cells is shown, scale bar = 100 µm. B) Zoomed in image of one GC, scale bar = 20 µm. Images courtesy of HF (BSc project, 2015) in collaboration with KT and JM.

The positive result for AR protein expression in cultured GCs at 48 hours, led to the question whether these cells were responsive to androgen signalling. Cells were cultured for 24 or 48 hours with DHT (10 nM), then collected for RNA extraction and investigation into variations in androgen responsive genes. At 24 hours, there was an increase in *Amh* and *Fshr* mRNA expression with DHT (Figure 8.6). At 48 hours the increase in *Fshr* was maintained, and there was an increase in *Star* mRNA levels. It has been shown previously that *Fshr* and *Star* transcription is increased in DHT treated follicles demonstrating that these cells behave similarly to intact preantral follicles in...
culture (see Figure 6.9) (Laird et al., 2017). No statistical analysis was performed on this data as only two biological samples were analysed.

**Figure 8.6 GCs exposed to DHT display changes in gene expression.**
GCs cultured in vehicle control or DHT (10 nM) for 24 or 48 hours. Gene expression data is displayed as fold change from control. Data shown is individual data points (n = 2 biological repeats, pooled GCs from 2 mice / 4 ovaries / repeat). Data courtesy of HF (BSc project, 2015) in collaboration with KT and JM.

In Chapter 6 it was shown that isolated preantral follicles in culture with DHT display a dramatic increase in AR protein expression (see Figure 6.11) and AR staining was quantitatively more nuclear (see Figure 6.13). The expression and localisation of AR was investigated in isolated GCs in vitro. GCs were cultured on coverslips for 48 hours with DHT and fixed for immunofluorescence staining. AR protein appeared more nuclear in cells exposed to DHT compared to control (Figure 8.7). This result highlights
that AR is likely acting through genomic signalling mechanisms in cultured GCs, however some AR staining did remain cytoplasmic in the DHT treated GCs.

Figure 8.7 GCs treated with DHT display increased nuclear AR protein expression. GCs cultured for 48 hours in vehicle control or DHT (10 nM) were stained for AR (red) and counterstained with nuclei label DAPI (blue). A) Representative image of multiple cells in control and DHT treatment are shown, scale bar = 50 µm. B) Zoomed in image of one GC in control and DHT treatment, scale bar = 10 µm. Images courtesy of HF (BSc project, 2015) in collaboration with KT and JM.

8.2.3 Rapid phosphorylation of ERK1/2 is detected post DHT treatment

Non-genomic steroid signalling can elicit changes in phosphorylation states of second messenger signalling molecules within seconds or minutes post-stimulation. In order to examine the presence of non-genomic androgen signalling in developing follicles, GCs were cultured for 48 hours and treated with DHT (10nM) for short time points, then lysed in RIPA buffer for protein extraction. Phosphorylated proteins were analysed through western blotting. At 2 minutes post-treatment there was an increase in ERK1/2 phosphorylation with DHT (Figure 8.8). This stimulation was reduced and not
significant by 5 minutes and lost by 10 minutes. There was no detectable response in the phosphorylation of AKT following DHT treatment at any time point examined.

**Figure 8.8 DHT stimulates rapid phosphorylation of ERK1/2 at 2 minutes.**
A) Representative blots showing ERK1/2 and AKT phosphorylation in cultured GCs treated with either vehicle control or DHT (10 nM) for 2, 5 and 10 minutes. B) Quantification of ERK1/2 and AKT phosphorylation in GCs treated with DHT. Signal intensity calculated relative to calnexin as internal loading control, and justified to control sample within time points (n = 5 biological repeats). Bars shown are mean ± SEM, statistical analysed by unpaired t-test within time points, where *** p < 0.001.

In order to elucidate the signalling mechanisms responsible for the rapid phosphorylation events observed with DHT, various pathway inhibitors were employed (Figure 8.9). Cells were pre-treated with inhibitors for 2 hours before stimulation with DHT for 2 minutes. Flutamide is a non-steroidal antiandrogen and acts as a selective antagonist to inhibit signalling through AR. ErbB activity was inhibited with either AG1478, a selective tyrosine kinase inhibitor against EGFR or ErbB2 inhibitor ii, a small molecule inhibitor which blocks ErbB2 phosphorylation. The Src-family of kinases has been implicated in relaying the non-genomic AR response in the cytoplasm of Sertoli cells (Cheng et al., 2007a). Therefore, PP2, an inhibitor of the Src family of kinases, was
also used. The 10 µM concentration of PP2 was chosen based on previous studies and dose responses performed in mouse ovarian tissue, which found 10 µM PP2 was maximally effective in theca-interstitial cell cultures (Chaturvedi et al., 2008). MEK1/2 activity was inhibited with U0126 as before, and finally PI3K signalling was inhibited with LY294002. Doses of U0126 and LY294002 were based on previous work in Chapters 4 and 7.

Figure 8.9 Hypothesised pathways of non-genomic AR signalling involving the ErbBs and the inhibitors employed.
Mouse GC cells were pre-treated with various pathway inhibitors including; flutamide (AR inhibitor), AG1478 (EGFR inhibitor), ErbB2 inhibitor ii (ErbB2 inhibitor), PP2 (Src kinase inhibitor), U0126 (MEK1/2 inhibitor) and LY294002 (PI3K inhibitor), then treated with dihydrotestosterone (DHT) for short times points and phosphorylation levels of ERK1/2 and AKT were measured. Dotted line represents proposed mechanisms of non-genomic AR signalling involving ErbBs. Turquoise receptor represents ErbB dimerisation partner. AR, androgen receptor; EGF, epidermal growth factor; MEK, Mitogen-activated protein kinase kinase; PI3K, Phosphatidylinositol 3-kinase.
Pre-treatment with either flutamide, AG1478 or U0126 significantly attenuated the peak in ERK1/2 phosphorylation seen with DHT exposure (Figure 8.10). This reduction may indicate that AR, EGFR and MEK1/2 are involved in the non-genomic androgen signalling pathway in mouse GCs. However, it is important to note that this is only preliminary data, as the inhibitors were not employed alone. U0126 inhibited baseline phosphorylation below that of control, suggesting that the MAPK pathway is active in untreated cultured GCs and highlights the importance of including ‘inhibitor alone’ controls. This extra control needs to be included in future experiments to accurately draw conclusions from these results. There was no decrease in DHT stimulated phosphorylation with ErbB2 inhibitor ii. Interestingly, inhibition of the Src-family of kinases did not ablate the DHT response, which may indicate that unlike Sertoli cells, the Src kinases may not be responsible for relaying the non-genomic androgen response in GCs. Again, there was no change observed in AKT phosphorylation with DHT, however baseline phosphorylation was inhibited below control by PP2 and LY294002. This suggests that the PI3K/AKT pathway is active in untreated GCs and again highlights the necessity for an inhibitor alone control.
Figure 8.10 DHT-induced rapid phosphorylation of ERK1/2 and AKT with pathway inhibitors.

A) Representative blots showing ERK1/2 and AKT phosphorylation in cultured GCs. Cells were pre-treated with vehicle control, flutamide (20 μM), AG1478 (10 μM), ErbB2 inhibitor ii (20 μM), PP2 (10 μM), U0126 (10 μM) or LY294002 (10 μM) for 2 hours, followed by DHT (10 nM), vehicle control or positive control (10 ng/ml EGF) for 2 minutes.

B) Quantification of ERK1/2 and AKT phosphorylation. Signal intensity calculated relative to calnexin as internal loading control, and justified to control sample (n = 3 biological repeats). Bars shown are mean ± SEM, statistical analysed by one-way ANOVA with Bonferroni multiple comparison test, where * p < 0.05, ** p < 0.01, *** P < 0.001.

8.2.4 DHT stimulates rapid phosphorylation of ERK1/2 in human GL cells

Following on from the work performed in the mouse which demonstrated the presence of non-genomic androgen signalling within GCs from developing follicles (see Figure
a similar study was performed in human GL cells to explore the presence of non-genomic androgen signalling in the human ovary.

GL cells were isolated and cultured for 48 hours, serum starved overnight and treated for 15 minutes with DHT at 10 nM and 100 nM. At 10 nM DHT significantly induced rapid ERK1/2 phosphorylation in the human GL cells (Figure 8.11). There was no further increase with 100 nM DHT. Again, no significant elevation was measured in AKT phosphorylation following androgen treatment. Samples were divided into normal morphology ovaries with regular cycles and women with polycystic ovaries with irregular cycles. Preliminary results show that women with polycystic ovaries with irregular cycles appear more responsive to rapid DHT treatment.

**Figure 8.11 DHT stimulates rapid phosphorylation of ERK1/2 in human GL cells.**

A) Representative blots showing ERK1/2 and AKT phosphorylation in cultured GL cells treated with either vehicle control or DHT (10 nM and 100 nM) for 15 minutes. B) Quantification of ERK1/2 and AKT phosphorylation in GL cells treated with DHT for 15 minutes. C) Quantification of ERK1/2 phosphorylation and D) AKT phosphorylation in GL cells separated into women with normal morphology ovaries and regular cycles (Normal / Reg) and women with polycystic ovaries and irregular cycles. Signal intensity was normalised to calnexin as internal loading control, and expressed as fold change relative to control (n = 5 combined, n = 3 Normal / Reg, n = 2 PCO / Irreg). Bars shown are mean ± SEM, statistical analysed by one-way ANOVA and Bonferroni multiple comparisons test, where * p < 0.05.
8.3 Discussion

8.3.1 Mouse GCs can be isolated and grown successfully as a monolayer and are responsive to androgens

A GC monolayer culture was established and optimised. Initial experiments showed evidence for spontaneous luteinisation during culture therefore several steps were taken to prevent GC differentiation. Optimal culture conditions included plating of GCs along with oocytes, maintaining cells in low serum content and reducing time in culture. Similar techniques have been shown to support the maintenance of the GC phenotype in other model species (Batta et al., 1980, Gutierrez et al., 1997a, Vanderhyden and Macdonald, 1998).

AR protein was detected in developing follicles in Chapter 6, peaking in the preantral follicle with lower expression observed at the antral stages of development. Additionally, AR protein expression is known to decrease in the preovulatory follicle and the CL, with AR reported absent from fully regressed CL in the primate ovary (Hild-Petito et al., 1991). As GCs were isolated from antral follicles from the d26 mouse ovary and were shown to luteinise over culture, AR protein expression was investigated. Cultured mouse GCs expressed both AR mRNA and protein after 48 hours in vitro, with AR protein localised to both the nucleus and the cytoplasm in untreated GCs. Upon DHT stimulation AR protein increased translocation to the cell nucleus, suggesting AR acts through genomic mechanisms to regulate gene transcription in cultured GCs. Indeed, androgen sensitive genes, Fshr and Star were both elevated in GCs exposed to DHT. However, there remained some cytoplasmic AR staining following DHT treatment, suggesting that not all AR signalling occurs within the nucleus.

8.3.2 DHT stimulates rapid phosphorylation of ERK1/2 in mouse GCs

Although DHT had been shown in Chapter 7 to require ErbB activity to maximally stimulate preantral follicle development, it was unclear if this was mediated via non-genomic signalling pathways. A key feature of non-genomic signalling is rapid response to ligand exposure, therefore in order to investigate the presence of non-genomic androgen signalling in developing follicles, primary GCs were cultured and treated with DHT for short time points then collected for protein analysis. At 2 minutes post-
stimulation DHT induced ERK1/2 phosphorylation. The speed of this stimulation cannot be a result of classical nuclear AR signalling, so strongly indicates the presence of non-genomic androgen signalling in mouse GCs from developing follicles. This result is supported by accumulating evidence in prostate cancer cells showing ERK1/2 phosphorylation occurring rapidly within one to two minutes of DHT treatment (Liao et al., 2013), peaks at 5 minutes recorded in breast cancer cells (Zhu et al., 1999), and within 2 minutes of stimulation in osteocyte-like cells (Kousteni et al., 2001). Additionally, studies performed in Sertoli cells, which are of particular interest, because they play a similar supporting role in the testis as GCs play in the ovary, phosphorylation of the transcription factor CREB and ERK1/2 increased by 2-fold within just 1 minute of androgen treatment (Fix et al., 2004).

Rapid stimulation of MAPK signalling with DHT in mouse GCs has been shown by one other study (Sen et al., 2014). This previous study however stimulated cells for 30 minutes. In contrast, the current study found that the DHT induced peak in ERK1/2 phosphorylation was lost by 10 minutes post-treatment. The reason for difference in timings is unclear but there may be multiple mechanisms at play mediating the non-genomic signal. Similar to our result, another study in 2d mouse ovaries found that stimulation of AKT phosphorylation with testosterone peaked at 5 minutes, was lessened at 15 minutes and lost by 30 minutes (Yang et al., 2010).

Several inhibitors were employed to elucidate the underlying mechanism of non-genomic signalling in developing follicles. The DHT induced peak in ERK1/2 phosphorylation was ablated by pre-treatment with flutamide (AR inhibitor), AG1478 (EGFR inhibitor) or U0126 (MEK1/2 inhibitor). Interestingly, there was no attenuation with ErbB2 inhibitor ii (ErbB2 inhibitor) or PP2 (Src inhibitor). However, it is important to note that the inhibitors were not employed alone. As the inhibitors suppressed baseline ERK1/2 and AKT phosphorylation below levels of control, conclusions cannot yet be accurately drawn from this data. Further experimentation is required to confirm the involvement of AR, EGFR and MEK1/2 in non-genomic androgen signalling pathways in mouse GCs.
Similarly, Sen et al., found that pre-treatment with either flutamide or AG1478 could significantly attenuate the DHT induced response in ERK1/2 phosphorylation in mouse GCs, implicating EGFR in the non-genomic pathway. Interestingly, they also found that pre-treatment with the MMP inhibitor Galardin ablated MAPK activation (Sen et al., 2014). The authors concluded that non-genomic AR signalling occurred via stimulation of MMP-mediated shedding of membrane bound EGFR ligands. However, contrasting results have been found in Sertoli cells. The addition of AG1478 also ablated rapid MAPK phosphorylation in Sertoli cells, however, pre-treatment with either a MMP inhibitor, an antibody blocking EGFR ligand binding or antiserum against HB-EGF did not reduce testosterone-induced ERK or CREB phosphorylation (Cheng et al., 2007a). The authors hypothesised that non-genomic AR signalling occurred via intracellular stimulation of EGFR in Sertoli cells without liberation of EGF-like ligands. The mechanism by which AR could activate EGFR in the current study is unknown, but may involve either extracellular MMP-mediated shedding of membrane bound EGF-like ligands, or intracellular interaction with intermediate kinases. Future experiments could investigate the accumulation of EGF-like ligands in culture medium following DHT treatment.

The cytoplasmic kinase Src has been implicated in androgen non-genomic signalling in many cell types. Studies in Sertoli cells found an increase in AR-Src protein interactions at the plasma membrane following testosterone treatment from 5 minutes, shown by immunoprecipitation of Src (Cheng et al., 2007a). Furthermore, addition of a Src kinase inhibitor ablated androgen induced rapid MAPK signalling. Additionally, rapid ERK1/2 phosphorylation detected within 2 minutes of androgen stimulation in osteocyte-like cells was blocked with the addition of Src kinase inhibitor PP1 (Kousteni et al., 2001). The present study however, found no decrease in DHT stimulated ERK1/2 phosphorylation when pre-treated with PP2 a broad-spectrum inhibitor of Src. Future studies could examine the phosphorylation status of Src following androgen treatment, or use confocal microscopy to examine AR-Src interactions at the plasma membrane, as used successfully in Sertoli cell cultures.

The peak in MAPK phosphorylation with DHT was short-lived, with significant elevations lost by 5 minutes. The brief duration of the signal may raise uncertainty into
the biological relevance of this signalling pathway in developing follicles, which is still unknown. The classical genomic model of AR signalling is likely responsible for many of the changes seen with DHT in developing follicle, while the physiological role of non-genomic AR signalling is less clear. Due to the likely crosstalk and convergence between the genomic and non-genomic signalling pathways it is extremely difficult to determine the contribution of the non-genomic pathway in developing follicles. However non-genomic signalling could help to explain androgen induced modifications in transcription of genes that do not possess an ARE in their promoter region. A study performed in 2d mouse ovaries has postulated a role of non-genomic androgen signalling during follicle activation (Yang et al., 2010). The authors found that rapid phosphorylation of AKT with testosterone treatment resulted in Foxo3a phosphorylation and translocation from the nucleus after 30 minutes. Foxo3a exclusion from the oocyte nuclei has been shown to be a critical step in primordial follicle activation (Castrillon et al., 2003). Androgens have been shown previously by our group and others to promote follicle activation in vitro (Laird, unpublished) and in vivo (Abbott et al., 1998, Vendola et al., 1998), and therefore, may act through both genomic and non-genomic mechanisms to do so. The current study found no change in AKT phosphorylation following DHT treatment, however difference in follicular stage may explain this finding. The d2 mouse ovary contains predominantly primordial follicles with few growing follicles. AR has been localised to the oocytes of primordial follicles (see Chapter 6) and thus AR may be acting via another mechanism to elicit non-genomic signalling in the oocyte rather than GCs isolated from growing follicles.

To investigate the outputs of non-genomic signalling in GCs from developing follicles one option is to investigate activation of downstream transcription factors known to be activated by MAPK such as CREB. CREB binds to the cAMP-response elements (CRE), a sequence identified in the promoters of multiple inducible genes, to regulate transcription. Phosphorylation of CREB was found after 1 minute of androgen stimulation in Sertoli cells (Fix et al., 2004) and should be investigated in mouse GCs in future studies.

Others have attempted to dissect the contribution of non-genomic signalling by the generation of pathway-specific androgen receptor mutants. The ARC562G mutation has
one amino acid change that disrupts the structure of a zinc finger, the resultant AR structure is incapable of binding to DNA. In CV-1 cells, cells expressing the ARC562G mutant AR displayed reduced transcriptional activity (Yarbrough et al., 1990), demonstrating a block in classical AR genomic signalling. Alternatively, ARΔ372–385 mutant AR has been shown to retain the ability to activate AR-mediated transcription but lacks a proline-rich domain required to interact with c-Src, and therefore cannot activate Src mediated non-genomic signalling (Migliaccio et al., 2007). Studies using Sertoli cells isolated from tfm rats, successfully transfected cells with vectors expressing the ARC562G or ARΔ372–385 mutant forms of AR or the wild-type AR, as well as a luciferase reporter plasmid driven by the AR-inducible prostate-specific antigen (PSA) promoter (Shupe et al., 2011). It was found that while the ARC562G mutant cells were incapable of activating the PSA promoter, the ARΔ372–385 mutant or overexpressing wild-type AR could both successfully induce the promoter. The authors went on to investigate germ cell attachment and found that Sertoli cells overexpressing the wild-type AR or ARC562G mutant displayed approximately a doubling of germ cells attached in the presence of testosterone, whereas quantities of germ cells attached were not increased after testosterone stimulation of Sertoli cells overexpressing the ARΔ372–385 mutant. The authors hypothesised that these results showed that non-genomic signalling of testosterone contributes to Sertoli germ cell adhesion and that the DNA-binding activity of AR is not sufficient to permit testosterone-induced germ cell binding. It would be interesting to investigate if similar studies could be performed in the ovary.

8.3.3 DHT stimulates rapid phosphorylation of ERK1/2 in human GL cells

The rapid phosphorylation of ERK1/2 in response to DHT was replicated in the human GL cells, which unlike the mouse GCs obtained from antral follicles, are postovulatory luteinized cells. There was a significant increase in ERK1/2 phosphorylation following 15 minutes of DHT treatment, with no significant change in phospho-AKT, similar to the results obtained in the mouse. This result suggests that non-genomic androgen signalling may be present and play a role during both the early stages of follicular development and as well in the later post-ovulation luteinising GCs. Androgens have previously been implicated in the final stages of follicle development. For example administration of DHT increases ovulation rate and CL numbers in pigs (Cardenas et al., 2002), and increased ovulated oocyte numbers in mice (Sen et al., 2014). The
mechanisms by which androgens regulate preovulatory follicle development and ovulation remain unknown, however there is some evidence that testosterone promotes \textit{in vitro} germinal vesicle breakdown in mouse oocytes independent of transcription (Gill et al., 2004). However, little investigation has been undertaken in the surrounding cumulus GCs and should be investigated further.

\textbf{8.4 Conclusions}

The rapid phosphorylation of ERK1/2 in both human and mouse GCs with DHT treatment demonstrated that AR can signal through non-genomic pathways in the ovary. However, it is extremely difficult to dissect out the relative importance of non-genomic and genomic signalling in the developing follicle, and there is likely much overlap. Follicle culture studies shown in Chapter 7 with DHT added in combination with ErbB and MAPK inhibitors revealed a decrease in androgen induced follicle growth and may give an indication of the role of non-genomic signalling involving EGFR/ErbB2 in preantral follicle growth. However, these studies collected data over a matter of days, making it difficult to determine the contribution of non-genomic signalling. Although the physiological significance of this signalling remains unclear, these non-classical pathways may support and enhance classic AR signalling, as well as activating additional transcription factors to regulate gene expression and promote follicle growth.
Chapter 9 Summary and conclusions

The tight regulation of preantral follicle development within the mammalian ovary is critical in reproductive health and fertility. The steady rate of primordial follicle activation and preantral follicle growth must be carefully orchestrated to ensure a healthy supply of oocytes for the entirety of the reproductive lifespan. Despite this, the precise mechanisms controlling these early developmental stages remain elusive. As the immature preantral follicle develops in a largely avascular environment, local intraovarian growth factor, cytokine and steroid signalling pathways likely play a central role in the regulation of growth and survival. This thesis has provided evidence for both the EGF family of growth factors and androgenic steroids as important regulators of early development. These factors likely contribute to a complex and interconnected regulatory network that exists within the ovary. This thesis first examined the individual effects of the EGF family and androgens on preantral follicle development, then explored evidence for crosstalk between the two families.

The essential role for the EGF family during ovulation is well characterised (Conti et al., 2006), however the role of EGF signalling during the preantral stages of follicle development has been largely overlooked. This thesis has shown that ErbB receptor subtypes EGFR and ErbB2 are both present in mouse preantral follicles at both the mRNA and protein level. As the early stages of follicle development rely on local factors, EGF-like ligands were also investigated, finding TGF-α and EGF present in the oocyte and GCs of preantral follicles, as well as mRNA for HB-EGF, Btc, Areg and Ereg detected in isolated GCs. Future work should investigate the protein expression of the EGF-like ligands as well as their cleavage from membrane bound precursors to assess their availability in preantral follicles. These results demonstrate that EGF-ligands produced locally within the GCs and oocyte may signal through the ErbBs expressed in neighbouring preantral follicles or within the follicle itself to regulate development. Interestingly, when EGFR was selectively inhibited with AG1478 in our preantral follicle culture system, growth was significantly reduced below control, indicating that EGFR is activated by factors endogenous to the isolated preantral follicle.
In the neonatal mouse ovary, predominantly populated by dormant primordial follicles, the EGFR subtype was absent. Consistent with this finding, exogenous EGF added to neonatal ovary cultures showed no change in primordial follicle activation. EGF-like ligands and EGFR likely do not directly regulate primordial follicle activation, however they may indirectly. EGF treatment of preantral follicles caused a dramatic decrease in AMH mRNA and protein expression, a well characterised inhibitor of primordial follicle activation produced by growing follicles (Durlinger et al., 2002b). Further studies should examine the effects of EGF on cultured ovary fragments containing both primordial and larger preantral follicles. In contrast, ErbB2 protein was present in the neonatal ovary, however as ErbB2 lacks a known ligand it requires other ErbB receptor subtypes to form heterodimers to signal. The role of ErbB2 in the neonatal ovary is therefore unclear. However, immunohistochemistry revealed a remarkably similar spatial localisation pattern to adherens junctions in the transitional follicle immediately after activation (Mora et al., 2012). The EGF family and ErbB2 may play a role in GC morphological changes during follicle activation and the partial EMT accompanying follicle growth, as EGF was shown to regulate the expression of N-cadherin in preantral follicles in culture. Future studies should employ inhibitors of ErbB2 during neonatal ovary culture and examine changes in activation rate, expression of adherens junctions as well as GC morphology.

In the isolated multi-layered preantral follicle EGFR and ErbB2 subtypes were both expressed. ErbB2 protein expression peaked in the GCs of the primary follicle and decreased as the follicle began to multilayer, indicating its importance during the earliest stages of preantral follicle development. Although EGFR has not yet been immunolocalised, preliminary western blots show that EGFR is expressed in the GCs of developing follicles. Exogenous EGF added to individual follicle cultures caused a significant increase in GC proliferation and preantral follicle size. This suggests that EGF and EGFR signalling may switch on after activation to regulate the progression of preantral follicle development. A drawback of the preantral follicle culture is that any effects from surrounding stroma and theca are removed. As EGFR protein may be present in the stroma it would be interesting to also observe the effects of EGF in the recently developed ovarian fragment cultures. The co-expression of EGFR and ErbB2 indicates that both EGFR homodimers and EGFR-ErbB2 heterodimers likely form upon
EGF stimulation. Indeed, selective inhibition of either EGFR with AG1478 or ErbB2 with ErbB2 inhibitor ii significantly attenuated the stimulatory effects of EGF on follicle growth. Furthermore, it appears the MAPK pathways is activated downstream of the ErbBs and is responsible for the mitogenic response as inhibition of MEK1/2 with U0126 also abolished the effect of EGF. As well as stimulating follicle growth, EGF treatment has wide-ranging effects on genes and proteins central to the regulation of preantral follicle development. Of particular interest, EGF causes down-regulation of an array of growth factor ligands and receptors including those in the TGF-β superfamily, such as GDF9, BMP15 and TGFβ2, known to be essential regulators of primordial and preantral follicle development (Knight and Glister, 2006), as well as those in the EGF family, TGF-α and HB-EGF. It is likely that a complex network of local growth factors interacts to create a delicate balance of both stimulatory and inhibitory signals to control follicle growth, and therefore artificially increasing EGF stimulation causes the consequential reduction seen in the expression of other growth factors. Interestingly, EGF increased AR protein expression, giving the first indication of a crosstalk between the two families.

The EGF family has a clear role to play in preantral follicle growth and progression, before its requirement at ovulation. However, it is difficult to determine the redundancy within the system as very few in vivo studies have examined the role of these growth factors on follicle development and knock out models of the ErbBs are embryonic lethal. Further work could attempt to investigate the effects of ovarian EGF signalling in vivo, for example through the generation of ovarian specific ErbB knockout mice. EGF appears to have an extensive regulatory role on factors involved in proliferation, cell adhesion and steroidogenesis. However, the factors that regulate the EGF family are currently unknown. A hypothesis of this thesis was that androgenic steroids act within the preantral follicle to modulate growth factor signalling. This thesis has shown that local EGF signalling regulates preantral follicle development independently but consistent with this hypothesis, also interacts androgen signalling.

The androgen receptor is expressed throughout preantral follicle development, localised to the oocyte of the primordial follicle and within the GCs of growing follicles, while bioactive androgens are present in the circulation but are also produced locally by...
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ovarian theca cells. Ligand activated AR acts classically as a nuclear receptor to regulate gene transcription, however this thesis has shown that AR can also induce rapid (minutes, rather than hours) phosphorylation events in second messenger pathways such as the MAPK cascade in mouse GCs from developing follicles and human GL cells. The mechanism of this non-genomic signalling remains unclear but preliminary evidence implicates AR and EGFR in mouse GCs. Future work should use pathway inhibitors alone and in combination with short androgen treatment, and investigate phosphorylation of multiple second messenger targets in order to dissect out the mechanisms of non-genomic signalling in mouse GCs. Staining for AR protein became increasingly nuclear in response to DHT treatment in isolated preantral follicles and cultured mouse GCs, indicating that AR is acting through genomic mechanisms to regulate gene transcription, however, a portion of AR protein remained cytoplasmic, which may contribute to the non-genomic response. The term ‘non-genomic’ is misleading as cytoplasmic activated signalling cascades can also converge on the nucleus to regulate gene transcription and have been suggested by some to even support the nuclear actions of AR (Hammes and Davis, 2015). The brevity of the signal detected in MAPK phosphorylation, which was lost by 5 minutes, casts uncertainty over the biological consequences and relevance of non-genomic signalling in developing follicles. Due to the likely crosstalk and convergence between the genomic and non-genomic signalling pathways determining the relative contribution of each is extremely difficult. Further studies may examine phosphorylation of downstream transcription factors of MAPK such as CREB, or liaison proteins such as paxillin, to elucidate the signalling consequences of non-genomic AR pathways. This relatively unexplored mechanism of signalling in developing follicles may contribute to our understanding of androgen actions in the ovary in future studies.

This thesis has contributed to the body of evidence implicating androgens in preantral follicle development. Similar to previous studies, treatment with the potent non-aromatisable androgen DHT significantly increased GC proliferation and preantral follicle size. One novel observation was that DHT stimulated premature antrum formation in cultured mouse follicles. It has been postulated that androgens may regulate the expression of aquaporins, which facilitate water transport and play a role in the accumulation of fluid during antrum formation (Grzesiak et al., 2016). Future
studies should look at the expression of aquaporins present in mice GCs with androgen treatment. In contrast to previous reports, DHT treatment did not promote follicle survival, however our cultures are relatively short and further studies could investigate longer durations. Key regulators of preantral follicle growth were altered with DHT treatment, including AMH. Perturbed AMH expression has been linked to ovulatory disorders such as PCOS (Pigny et al., 2003), and has been used as a marker of the ovarian reserve. This thesis found a paradoxical decrease in AMH mRNA but increase in AMH protein expression with DHT, which may have consequences for primordial follicle activation in vivo. Understanding the complex interactions between androgen and AMH expression in the ovary may contribute to our understanding of disordered early follicle development in PCOS. Positive feedback of androgen signalling was evident in preantral follicles, as DHT exposure caused a dramatic increase in AR protein expression, consequently increasing sensitivity to androgens. This likely contributed to the sustained elevation in preantral follicle growth throughout culture. Increased androgen exposure in disorders such as PCOS, may increase the sensitivity of developing follicles to already high circulating and local androgens.

This thesis has provided evidence that androgens modulate local EGF signalling in developing preantral follicles. Androgen treatment altered the expression of both ErbBs and EGF-like ligands present in isolated follicles. DHT increased EGFR mRNA levels, the principal receptor responsible for binding EGF-like ligands, and may therefore increase the sensitivity of follicles to ligands produced by neighbouring follicles or within the follicle itself. Due to technical constraints the protein expression of EGFR with DHT remains under investigation, however western blotting on follicles was optimised as part of this thesis and could be used to examine EGFR protein expression in future. A potential synergy between androgen and EGF signalling was investigated using a novel combination of EGF and DHT treatment added to preantral follicle cultures. The two ligands produced an additive effect on follicle growth. Interestingly, ErbB2 mRNA and protein expression were both decreased with androgen treatment. As the role of ErbB2 in developing follicles has not yet been fully explored, the consequence of this down-regulation remains unclear. However, alterations in the relative expression of ErbB receptors may alter dimer compositions available upon ligand stimulation and the signalling outputs generated. Contradictory to the initial hypothesis transcription of
TGF-α and Btc were both repressed with DHT treatment. However, EGF-like ligand availability is not only based on their transcription, so protein levels and post-translational shedding of these ligands should be investigated further.

Surprisingly, androgen stimulated follicle growth was significantly attenuated with the addition of selective EGFR and ErbB2 inhibitors. This result may link to DHT induced changes in expression of ErbBs and EGF-like ligands, which would alter EGF family signalling pathways in the preantral follicle. Alternatively, the decrease may suggest that androgens require the ErbBs to activate non-genomic pathways to enhance proliferation. Additionally, removal of MAPK signalling with the MEK1/2 inhibitor U0126 also attenuated DHT stimulated follicle growth, and may further indicate a role for non-genomic signalling. However, a major limitation of this study is that changes in growth occur over a matter of days, which makes drawing conclusions over the contribution of genomic and non-genomic pathways extremely difficult. In the future studies may focus on shorter time points for changes in proliferation. Androgen interaction with local growth factors has been shown previously with factors such as IGF (Vendola et al., 1999, Hickey et al., 2004). This thesis has shown that androgens not only regulate expression of EGF family members but also require their activity to produce a maximal effect on preantral follicle growth.

Uncovering the complex mechanisms which control early follicle development can help in the understanding of disorders such as premature ovarian insufficiency and polycystic ovary syndrome which both present with disruptions in early follicle development. POI affects approximately 1% of women and the majority of cases are idiopathic (Goswami and Conway, 2005). Accelerated activation of the primordial follicle pool has been proposed as a contributor to POI. Both EGF and DHT were shown to affect AMH expression, one of the key negative regulators of primordial follicle activation, and overexpression of either may upset the balance of stimulatory and inhibitory signals during primordial follicle activation. Advancing our knowledge of the complex regulatory networks may help direct research toward potential diagnostic, preventative or therapeutic treatments. There is growing consensus that ovulatory abnormalities seen in women with PCOS most likely originate during early preantral follicle development. Additionally, a multitude of in vitro and in vivo studies have
highlighted intra-ovarian androgen excess as a major contributing factor in the development of disordered early follicle development in PCOS (Walters, 2015). Androgen interaction with local EGF family members during preantral follicle development could provide new insights into androgen modulation of follicle development in vivo. Recent work from within our group has established a prenatally androgenised mouse model of PCOS (Laird, unpublished). Future work may investigate changes in EGF family members in the ovaries from these mice to explore evidence for EGF involvement in androgen action in vivo. Furthermore, uncovering factors that support preantral follicle survival and growth in vitro may contribute to optimising culture conditions during in vitro maturation and IVF treatment. For young girls undergoing chemotherapy treatment for cancer before the onset of puberty there are currently few options to preserve fertility later in life, however ovarian biopsies containing primordial follicles can be collected and cryopreserved. Understanding the complex regulatory mechanisms that promote the maturation of primordial follicles to the ovulatory stage of development in vitro may provide strategies to overcome infertility in future.

In conclusion, preantral follicle development is regulated by a complex and interconnected network of intraovarian growth factors and steroids. The presence of both EGF-like ligands and receptors in preantral follicles coupled with the response to EGF seen in growth as well as gene and protein expression strongly suggest a role for local EGF signalling within preantral follicles. Preantral follicle development is also stimulated by androgens, which modulates EGF family signalling. Androgens alter expression of EGF family members in the preantral follicle but also require their activity to stimulate maximal effect on preantral follicle growth. This thesis has shown evidence for a crosstalk between androgen and EGF signalling families in preantral follicles, providing novel insights into the elusive regulatory mechanisms of early follicle development.


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